BATCH AND CONTINUOUS CULTIVATION OF ESCHERICHIA COLI

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BY
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CERTIFICATE

It is certified that this work has been carried out under my supervision and that this has not been submitted elsewhere for a degree.

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Date: 6th March 1970

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Satish L. Gandhi

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NOMENCLATURE

a_p, a_s, a_T, a_{T1}, b_s	Stoichiometric Constants
C	Dry Weight, gms.litre^{-1}
C_s	Concentration of Growth Limiting Substrate, gms./litre^{-1}
C_T	Concentration of Inhibitor, gms.litre^{-1}
C_v	Active Biomass Concentration, gms.litre^{-1}
D	Dilution Rate, hr^{-1}
K	Second order rate constant, litre gram^{-1}hr^{-1}
K_s	Michaelis-Menten Constant, gram litre^{-1}
K_j	Constant, gram litre^{-1}
k_1, k_2	First Order Rate Constants, hr^{-1}
k_3, k_4	Second Order Rate Constants, litre gram^{-1}hr^{-1}
N	Dead Protoplasmic Mass
P	Endogenous Substrate
S	Exogenous Substrate
T	Inhibitor
t	Time, hr.
V	Active Biomass or Viable Biomass
V'	Degradation Product
Y	Yield Coefficient, dimensionless
α	Stoichiometric Constant
μ	Specific Growth Rate, hr^{-1}
μ_m	Maximum Specific Growth Rate, hr^{-1}

Subscripts

ss	Steady State
i	Initial Condition

* * *

ABSTRACT

A continuous culture apparatus for the cultivation of Escherichia coli has been set-up. Batch and continuous culture studies of the growth of Escherichia coli have been carried out in a chemically defined medium with glucose as the sole limiting substrate.

The experimental data on the growth of E. coli has been presented for two different initial glucose concentrations of 2 gms./litre and 10 gms./litre. It has been found that there is a qualitative agreement between the experimental results and those predicted by Monod's model at the initial glucose concentration of 2 gms./litre. However, at the higher glucose concentration of 10 gms./litre, the Monod model predicts results widely different from those observed experimentally.

An attempt has been made to explain the observed experimental results qualitatively in the light of more realistic kinetic mathematical models proposed by other workers in this field.

CHAPTER - I

INTRODUCTION

The study of microbial cell populations is important because of the ability of microorganisms to produce a variety of complex products during growth. These products include proteins, antibiotics, amino acids, substances for insect control etc., and are of great economic importance to man. The study of microbial population will yield information about the various factors controlling growth of microorganisms which is an essential prerequisite for the analysis and design of processes in which microbial activity yields industrially important products.

Microbial cell populations can be studied in a batch or a continuous system. The study of microbial growth in a continuous system has a distinct advantage over that of a batch system. In a batch culture, the environmental conditions (substrate concentration, pH etc.) and physiological conditions are continuously changing, whereas, in a continuous culture they can be maintained at constant values with respect to time. The effect of different system parameters like dilution rate, substrate concentration etc., on the growth of microorganisms can then be studied under constant environmental conditions.

Though the continuous culture provides a better tool for the study of microbiological systems, several phenomena observed in a batch culture must somehow manifest themselves in a continuous culture. The validity of a kinetic mathematical model for microbial

growth will, therefore, depend on its ability to explain both batch and continuous culture results.

From an industrial point of view, the advantages of continuous processes over batch processes have been well established. To enumerate a few examples, continuous processes are characterized by their high production rate, are capable of turning out a product of more uniform quality, require little supervision and are well suited for the application of automatic control.

However, it must be pointed out that continuous processes though more rewarding, are also more exacting than batch processes in several ways. The situation in the microbiological industry today is analogous to that existing formerly in the chemical industry when the change from batch to continuous processes was beginning. It was then realized that more information was needed about fundamentals, for example, kinetics and thermodynamics of process reactions, chemical engineering problems relating to the flow of materials through processing units, etc.. A similar approach towards the study of microbial populations is essential. Batch and continuous culture experiments will be valuable in gaining a better understanding of the kinetics of the various processes involved during microbial growth. These experiments can be properly designed to test the validity of the kinetic mathematical models. Such study will be helpful in overcoming the often empirical and semi-empirical approach presently being used in the application of continuous culture methods in the microbiological industry.

The theoretical aspects of continuous cultivation were first considered by Monod[1,2] and Novick and Szilard[3] and subsequently discussed by Herbert, Elsworth and Telling[4]. Koga and Humphrey[5] have used the Monod model in the analysis of continuous cultures. The Monod model, however, is inadequate to explain all the different phases observed in batch growth.

Ramkrishna et.al.[6] have introduced the effect of formation of inhibitory products of metabolism during growth. They have formulated two types of model, the unstructured distributed model and the structured distributed model. The former predicts all the phases of batch growth except the lag phase, whereas the latter predicts the lag phase also as they take into account the past history of the cells.

Ramkrishna et.al.[7] have also proposed mathematical models based on the phenomenon of endogenous metabolism. These models are characterized by the following assumptions: The viable mass increases in amount by utilizing the exogenous substrate and simultaneously accumulates internal food reserves known as endogenous substrate. Both exogenous and endogenous substrates are used to preserve the viability of cells during growth. When the exogenous substrate is exhausted, growth comes to an end and endogenous substrate is utilized for the maintenance of viability. When the endogenous substrate is exhausted the viable cells die off. It can be easily seen that the above phenomenon can explain the stationary phase and the phase of decline in batch growth. The lag phase is again explained by assigning the protoplasmic mass some structure, thus introducing the effect of past history of the

cells during growth. These different models proposed by Ramkrishna et.al.[6,7] also indicate that inhibitory effects may become predominant only at higher substrate concentrations; at relatively low substrate concentrations the endogenous metabolism may have a limiting influence on growth.

Yano and Koga[8] and Andrews[9] have independently studied the dynamic behaviour of continuous culture subject to substrate inhibition at high concentration of the rate limiting substrate. They have modified the Monod equation for specific growth rate by incorporating in it the effect of substrate inhibition which has been well studied in enzyme reactions.

Kono and Asai[10,11] have studied the kinetics of batch and continuous cultures by introducing the concepts of critical concentrations and coefficient of consumption activity. They divide the batch growth curve in different phases and characterize each phase by its coefficient of consumption activity and the critical cell concentrations at the boundary points between two phases.

Malek and Fencl[12] have given a detailed account of theoretical and methodological aspects of continuous culture of microorganisms. The subject has also been dealt with in the textbook by Aiba, Humphrey and Millis[13]. A comprehensive review of the various mathematical models of microbial cell populations has been presented by Tsuchiya, Fredrickson and Aris[14].

The dynamics of microbial cell populations has been studied theoretically but an experimental verification of the kinetic mathematical models is lacking in the literature. In this work,

a continuous culture apparatus has been set-up. Batch and continuous culture data on the growth of Escherichia coli have been collected. Due to a limited amount of experimental data, quantitative analysis of kinetic mathematical models discussed above, except the Monod model, was not possible. A qualitative explanation of the experimental results in the light of these models has been presented.

CHAPTER - II

EXPERIMENTAL SET-UP

A. Batch Cultivation

Batch experiments were carried out in 500 ml. conical flasks. A shaker was used for agitation of culture. The shaker speed was preadjusted at a level to give sufficient agitation of the culture in the flasks and care was taken that the culture did not wet the cotton used to plug the mouth of the conical flasks. The shaker used could accommodate about 17 conical flasks of 500 ml. capacity or about 35 conical flasks of 50 ml. capacity. It was placed in a thermostatic chamber whose temperature could be set at any desired value between 20°C to 40°C. In the actual experiments the temperature was set at 35°C always.

B. Continuous Cultivation

The continuous culture apparatus consisted of the following parts:

a. Dosing Device

For very small flow rates as required in a laboratory scale unit, the dosing device must be exact, uniform and reproducible.

The dosing device used was based on the principle of the Mariotte flask serving as the reservoir of the nutrient medium[12]. The liquid flows from the storage vessel under constant hydrostatic pressure. This system is very sensitive to changes of environmental pressure and temperature. The device was, therefore, placed in a constant temperature room. The constancy of such small flow rates is also dependent on the method employed for the control of the

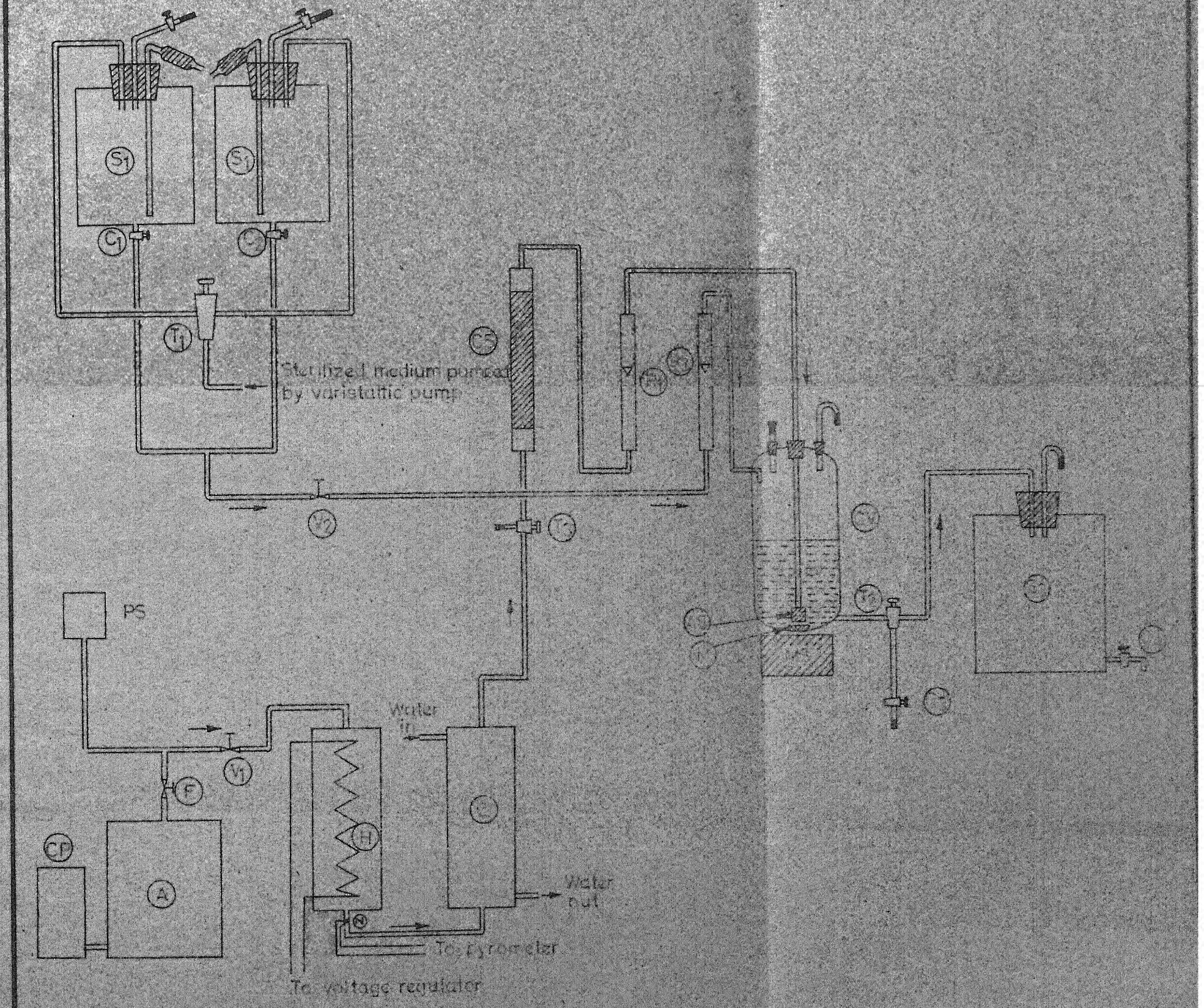

Fig 1 - Schematic diagram of apparatus.

FIGURE 1 - SYMBOLS

(S_1), (S_2)	Nutrient Medium Storage Tanks
(S_3)	Product Storage Tanks
(CV)	Culture Vessel
(CS)	Glass Wool Packed Sterilizer
(C_1),(C_2), (C_3),(C_4)	Two Ways Stop Cocks
(T_1), (T_2), (T_3)	Three Way Stop Cocks
(V_1), (V_2)	Stainless Steel Needle Valves
(F)	Flow Regulator
(PS)	Pressure Trip Switch
(R_1), (R_2)	Rotameters
(AS)	Air Sparger
(CP)	Air Compressor
(MS)	Magnetic Stirrer
(M)	Magnet
(A)	Air Storage Tank
(H)	Heater
(C)	Cooler
(N)	Thermocouple Junction

* * *

outflowing stream of the liquid nutrient medium from the storage flask to the culture vessel. In this set-up a stainless steel needle valve was used to control the flow rate, and the flow rate was measured by a glass rotameter placed after the needle valve. With this arrangement it was possible to vary the flow rate from 3 ml./minute to 30 ml./minute.

b. Pumping of Sterilized Nutrient Medium to the Storage Flasks

Two empty Mariottes (storage) flasks together with the flow control device were first dry sterilized in an oven before use. After sterilization, they were asceptically connected to the culture vessel by sterile rubber tubing. The storage flasks were then filled asceptically by sterilized nutrient medium with the help of a Manostat Varistaltic pump. This pump is ideal for handling sterile liquids. The pump is based on the principle that the fluid is pushed out by gradual flattening of the walls of an elastic tube. As soon as flattening ceases, the original shape of the tube is restored by its elasticity and powerful suction is created. Tygon tubing was used for pumping the fluid.

One stop-cock was provided at the outlet of each Mariotte flask and by using rubber tubing and a T-joint the outlets were connected with each other. All the connections have been shown in Figure 1. Only one stop-cock was kept open at a time for feeding the nutrient to the culture vessel. When this storage flask was empty, the stop-cock of the other storage flask was opened while that of the first closed. The empty storage flask was then asceptically filled with the fresh sterilized nutrient medium by the varistaltic pump. The above operations were repeated when the second flask was

empty and thus a continuous supply of sterilized nutrient medium at a fixed flow rate, as indicated by the rotameter, was maintained to the cultivator. A different flow rate could be obtained by simply adjusting the needle valve.

c. Culture Vessel

The culture vessel was made from 4 inch diameter pyrex glass tubing. It was 12 inches in height. The mouth of the culture vessel was made of a standard ground glass joint. The glass lid had four inlet tubes of about 1/2 inch in diameter. The nutrient medium was fed to the culture vessel from the side fitted with a standard glass joint, about 1 inch from the top. The outlet for the medium flow was situated at about 1 inch from the bottom. The central inlet tube at the top of the cultivator was used for air inlet, while the other three openings at the top, symmetrically located around the central tube, were used for air outlet, inoculation and temperature measurement.

The all-glass culture vessel is advantageous for laboratory work, enabling visual observation of cultivation, and is easy to sterilize and clean.

d. Maintenance of Constant Level in the Culture Vessel

For continuous cultivation studies the working volume of the culture vessel should be constant or in other words, the liquid level in the cultivator should be constant. In this set-up, it was achieved by connecting the outlet tubing of the culture vessel to the product receiver by three 90 degrees bends as shown in Figure 1. By this arrangement, it was also easy to change the

culture volume by simply changing the height of the glass tube connecting the culture vessel to the product receiver. The upper space above the liquid surfaces in the culture vessel and the product receiver was connected with the surrounding atmosphere through cotton wool filters placed in the air outlet lines, thus avoiding pressure variations in these vessels. This is necessary for maintaining a constant liquid level in the cultivator and also for a constant flow rate of nutrient to the cultivator.

e. Sampling

The sampling arrangement was made near the out-flow point of the culture vessel by using a three way stop-cock and another two way stop-cock near the sampling point, as shown in Figure 1. Whenever a sample was needed, the mouth of the sampling point was heat sterilized and the sample collected asceptically. The end of the sampling line was kept dipped in 70 percent alcohol solution in between two withdrawals.

f. Aeration and Agitation of the Culture

The continuous cultivation process requires perfect agitation of the culture so as to maintain a uniform concentration in the culture vessel. This was achieved by using a magnetic stirrer. The magnetic iron rod rotating on the bottom of the cultivator was protected by sealing into a teflon tube. As the culture vessel could be completely closed and sterilized together with the magnetic stirrer, it was well protected from outside contamination.

For this small scale laboratory set-up, however, aeration was found to be sufficient for the agitation of the liquid culture

as well. Hence, magnetic stirrer was not used in actual experiments. At the end of the aeration tube in the culture vessel was fixed a sintered glass sparger, which helped in uniformly distributing the air in the form of small bubbles throughout the culture and thus simultaneously providing efficient aeration and agitation. The air was supplied at the rate of 1.2 vol./vol.culture/minute.

A continuous supply of air at constant rate and pressure was available from a portable compressor working on a pressure-trip device. Two air storage tanks were connected to the outlet of the compressor. At the outlet of the air storage tanks, was attached a flow regulator. By using a pressure-trip device, the outlet pressure of the flow regulator was maintained within a very short range of 18 to 20 psig. as indicated by the pressure gauge. A needle valve was placed after the flow regulator to vary the flow rate of air. The air was then passed through a heater. The heater was provided with a heating element connected to a variable voltage supply. An 80 volts supply was found to be sufficient to raise the temperature of air to about 150°C. The air temperature was measured by connecting a thermocouple at the outlet end of the heater and connecting it to a pyrometer. The air was then passed through helical cooling coils, the coils being cooled by circulating water outside. The air then entered a pyrex-glass tube, about 18 inches long and 1 inch in diameter, packed with glass wool and the ends of which were closed by ground glass joints. A rotameter placed in the air line indicated the flow rate of air before bubbling the air through the sintered glass sparger in the culture vessel. With this arrangement it was possible to get sterilized air at constant flow rate, pressure and temperature.

g. Maintenance of Cultivation Temperature

The entire continuous culture set-up was placed in a thermostatic chamber. As the dosing device based on the principle of Mariotte flask is very sensitive to temperature changes, the thermostatic chamber facilities a constant flow of nutrient medium in the culture vessel, besides maintaining a constant temperature in the culture vessel. The temperature was kept at 35°C for all batch and continuous runs.

h. Sterilization

The entire continuous culture apparatus was sterilized by dry heat in an oven at 150°C for 24 hours.

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CHAPTER - III

EXPERIMENTAL METHODS AND RESULTS

A. Organism and Culture Medium

A pure culture of Escherichia coli strain B-26, supplied by the National Sugar Institute, Kanpur, was used throughout this study. The growth studies were carried out in a chemically defined medium of the following composition:

Di Ammonium Hydrogen Phosphate($(NH_4)_2HPO_4$)	2 gms.
Potassium Dihydrogen Phosphate (KH_2PO_4)	2 gms.
Sodium Chloride (NaCl)	4 gms.
Magnesium Sulphate ($Mg\ SO_4 \cdot 7H_2O$)	0.1 gms.
Glucose ($C_6H_{12}C_6$)	2 gms.
Distilled water	1000 ml.

The pH value of the nutrient medium before sterilization was adjusted to 7.4 by adding 5N NaOH solution. In this medium glucose was the sole carbon source and was also the growth limiting component, all other components being present in excess. For studying the effect of the growth limiting substrate on the growth of E.coli, the glucose concentration in the medium was varied, the concentration of other components remaining the same.

The composition of the nutrient agar medium used for preservation of the pure culture of E. coli and during plating for viable count was the same as the nutrient medium used for growth studies, The nutrient agar medium contained 1.5 percent of bacteriological agar. The nutrient medium was sterilized at 5 psig. for 30 minutes. When the glucose concentration was higher than 2 gms/litre, it was found to decompose on sterilization. It was

therefore, separately sterilized and added asceptically to the rest of the medium.

B. Operation

In batch cultivation, 125 ml. of nutrient medium was taken in 500 ml. flasks. Seventeen flasks were used for a single run. The flasks were sterilized and inoculated with 1 ml of a shake flask culture of E. coli grown on the same nutrient medium. The flasks were then placed in the shaker and growth was allowed to take place. The flasks were removed at definite intervals of time for complete analysis (see Appendix A). A batch run lasted for about six days.

In continuous culture studies, the entire apparatus was first dry heat sterilized in an oven at 150°C for 24 hours. The parts were then connected asceptically and mounted on the angle iron frame. The Mariotte flasks were filled with sterilized nutrient medium. The culture vessel was charged with 1 litre of sterile nutrient medium and inoculated with 1 ml. of a shake flask culture of E. coli. grown on the same medium. Growth was allowed to proceed batchwise until the concentration of the organisms was near the maximum value as shown by the turbidity measurements. Medium flow through the culture vessel was then started at a particular flow rate and the culture from then on run continuously. Samples of the culture were taken hourly and analyzed.

C. Observations and Measurements

Each sample from batch culture was analyzed for turbidity, dry weight, viable count/15/ and glucose concentration /16/. In one batch run total counts were also taken. The samples from

continuous culture runs were analyzed for turbidity, viable count and glucose concentration. The dry weight and total counts were then estimated from turbidity data using standard calibration curves obtained from batch runs.

The determination of turbidity, dry weight, total count, viable count and glucose concentration has been outlined in the Appendix A.

D. Experimental Results

Table I summarizes the experimental data on batch and continuous cultures. The values of maximum specific growth rate, yield coefficient and saturation constant in batch culture and steady state bacterial concentration, substrate concentration and yield coefficient in continuous culture are tabulated. Two sets of bach and continuous culture results were obtained with initial glucose concentration of 2 gms./litre and 10 gms./litre. The theoretical steady state values of bacterial and glucose concentrations were obtained from Monod's model using the values of maximum specific growth rate and yield coefficient obtained from batch culture and saturation constant from continuous culture (Appendix B). Determination of the saturation constant from batch culture experiments is difficult since at low substrate concentrations necessary for the estimation of specific growth rate, the value of glucose concentration is continuously decreasing and also glucose concentration being very low an accurate determination of its value is difficult. Saturation constant(K_s) was therefore estimated from continuous culture data with 2 gms./litre initial glucose concentration (Appendix B).

Batch culture experimental results on the growth of E.coli are presented in Tables II and III with initial glucose concentrations of 2 gms./litre and 10 gms./litre respectively. A single batch run lasted for 120 hours. The growth parameters measured as a function of time were glucose concentration, dry weight, viable count and optical density. Total counts measurements were also performed in the batch culture with 2 gms./litre initial glucose concentration. These values have been plotted in Figures 2 to 13.

Continuous culture experimental results on the growth of E. coli are presented in Tables IV and V with feed glucose concentration of 2 gms./litre and 10 gms./litre respectively. The results for 2 gms./litre feed glucose concentration have been obtained at two dilution rates of 0.36 hr^{-1} and 0.66 hr^{-1} and those for 10 gms/litre feed glucose concentration at a single dilution rate of 0.54 hr^{-1}. The continuous culture run at each dilution rate lasted for about 20 hours. The growth parameters measured as a function of time were glucose concentration, viable count and optical density. These results have been plotted in figures 14 to 22. The dry weights were estimated from calibration curves of dry weight versus optical density, obtained from batch culture experiments.

TABLE I

A COMPARISON OF EXPERIMENTAL RESULTS AND THOSE OBTAINED USING THE MONOD MODEL

	Experimental Batch Culture Data			Experimental Continuous Culture Data				Theoretical Continuous Culture Data Using Monod's Model	
C_{s1} (gms/lit)	Y	μ_m (hr^{-1})	K_s (mgms/lit.)	D (hr^{-1})	Y	$(C_x)_{ss}$ (gms/lit.)	$(C_s)_{ss}$ (gms/lit.)	$(C_x)_{ss}$ (gms/lit.)	$(C_s)_{ss}$ (gms/lit.)
2.0	0.399	0.76	15.06	0.36	0.348	0.687	0.024	0.793	0.0135
				0.66	0.332	0.655	0.029	0.758	0.0995
10.0	0.321	0.58	15.06	0.54	0.161	0.160	9.06	3.150	0.203

TABLE II

BATCH CULTURE DATA

C_{si} = 2 gms./litre

Sl. No.	Time t (hrs)	Optical Density	Dry Weight C (mgms/lit.)	Total Count x 10^{-7}	Viable Count x10^{-7}	Glucose Concentration, C_s (mgms/lit.
1	0	0.002	6	0.8	0.04	2000
2	2	0.002	6	0.8	0.12	1980
3	3	0.004	12	2.0	0.55	1950
4	4	0.005	16	3.5	0.63	1849
5	5	0.010	30	11	1.40	1730
6	6	0.026	78	36	11.2	1645
7	7	0.082	245	122	73	227
8	8	0.115	344	172	216	22
9	9	0.122	365	183	126	16
10	10	0.186	555	281	227	22.5
11	11	0.258	771	391	195	15.0
12	12	0.260	776	394	150	17.1
13	13	0.263	792	402	97	17.1
14	14	0.265	792	402	111	17.1
15	15	0.265	792	402	267	16.7
16	16	0.265	792	402	25	14.6
17	24	0.262	782	397	139	14.6
18	30	0.265	792	402	110	14.1
19	33	0.265	792	402	73	14.1
20	36	0.265	792	402	78	14.1

Table II (Contd)

21	41	0.265	792	402	89	14.1
22	48	0.265	792	402	117	14.1
23	52	0.245	732	371	186	14.1
24	70	0.255	762	386	75	14.1
25	84	0.255	762	386	161	14.1
26	96	0.235	702	355	120	14.1
27	108	0.245	732	371	111	14.1

* * *

TABLE III

BATCH CULTURE DATA

C_{si} = 10 gms/litre

Sl. No.	Time t (hrs)	Optical Density	Glucose Concentration C_s (gms/lit.)	Dry Weight C (mgms/lit.)	Viable Count x 10^{-7}
1	0	0.005	10.00	28	3
2	2	0.010	9.86	47	12
3	3	0.032	9.68	125	15
4	4	0.044	9.41	170	19.5
5	5	0.083	8.75	315	62
6	6	0.144	8.02	539	160
7	7	0.245	7.62	910	260
8	8	0.265	6.68	984	280
9	9	0.292	6.45	1082	330
10	10	0.292	6.28	1082	350
11	14	0.275	5.80	1020	350
12	18	0.290	4.82	1076	380
13	26	0.272	4.20	1009	360
14	34	0.294	3.32	1090	350
15	46	0.308	2.82	1011	-
16	70	0.330	2.72	954	-
17	96	0.370	2.42	907	-
18	120	0.390	2.42	921	-

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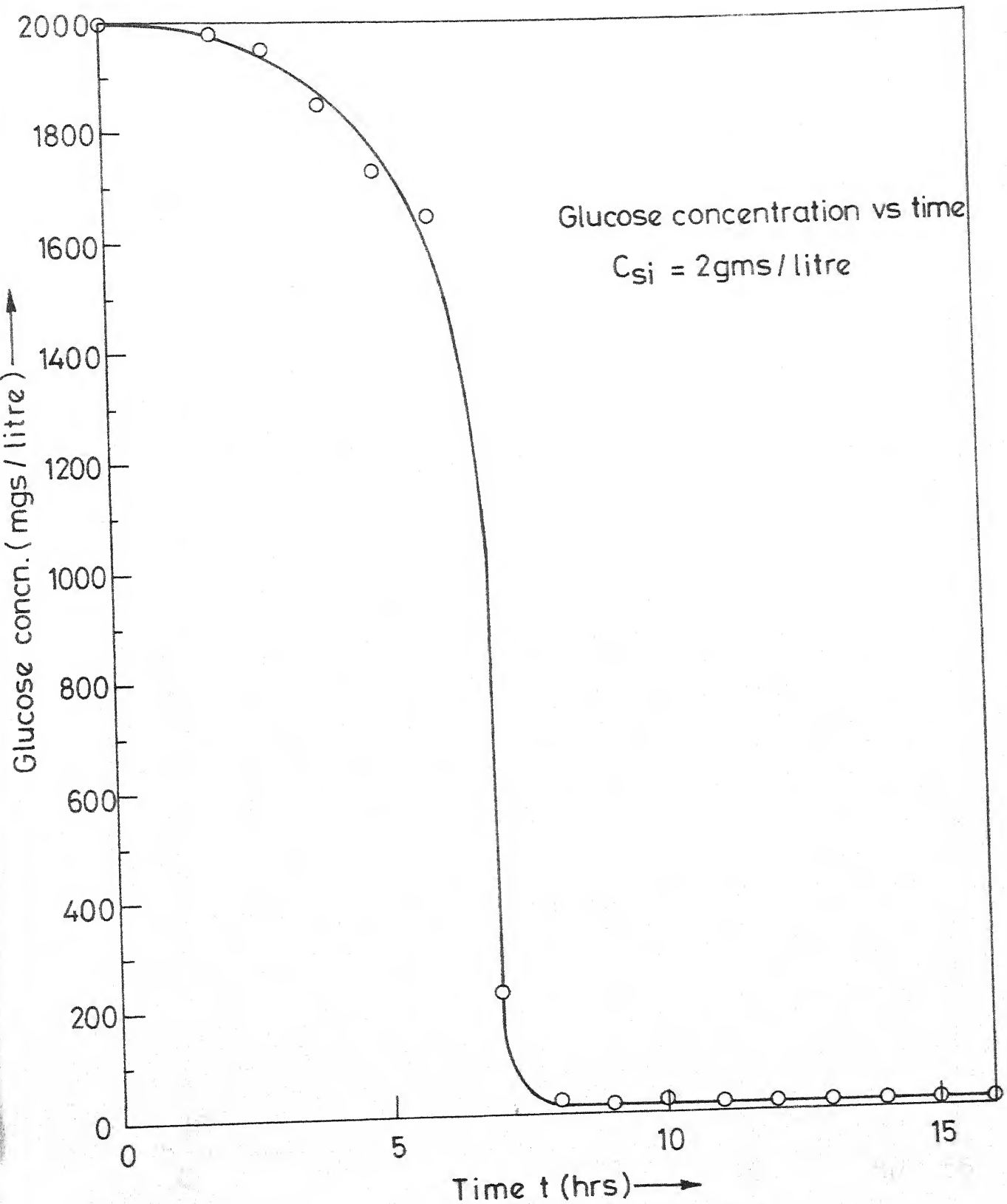


Fig. 2.- Batch culture.

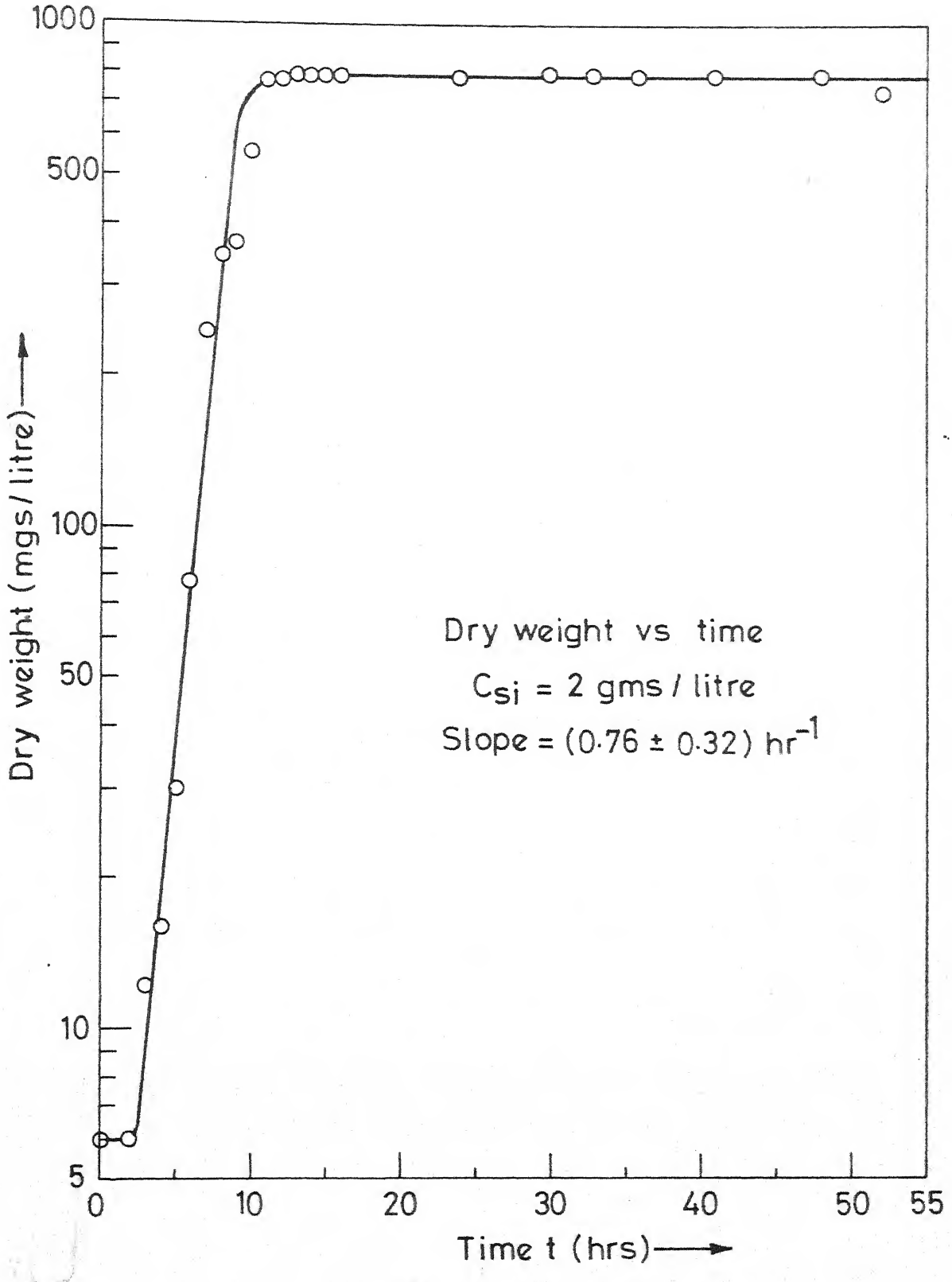
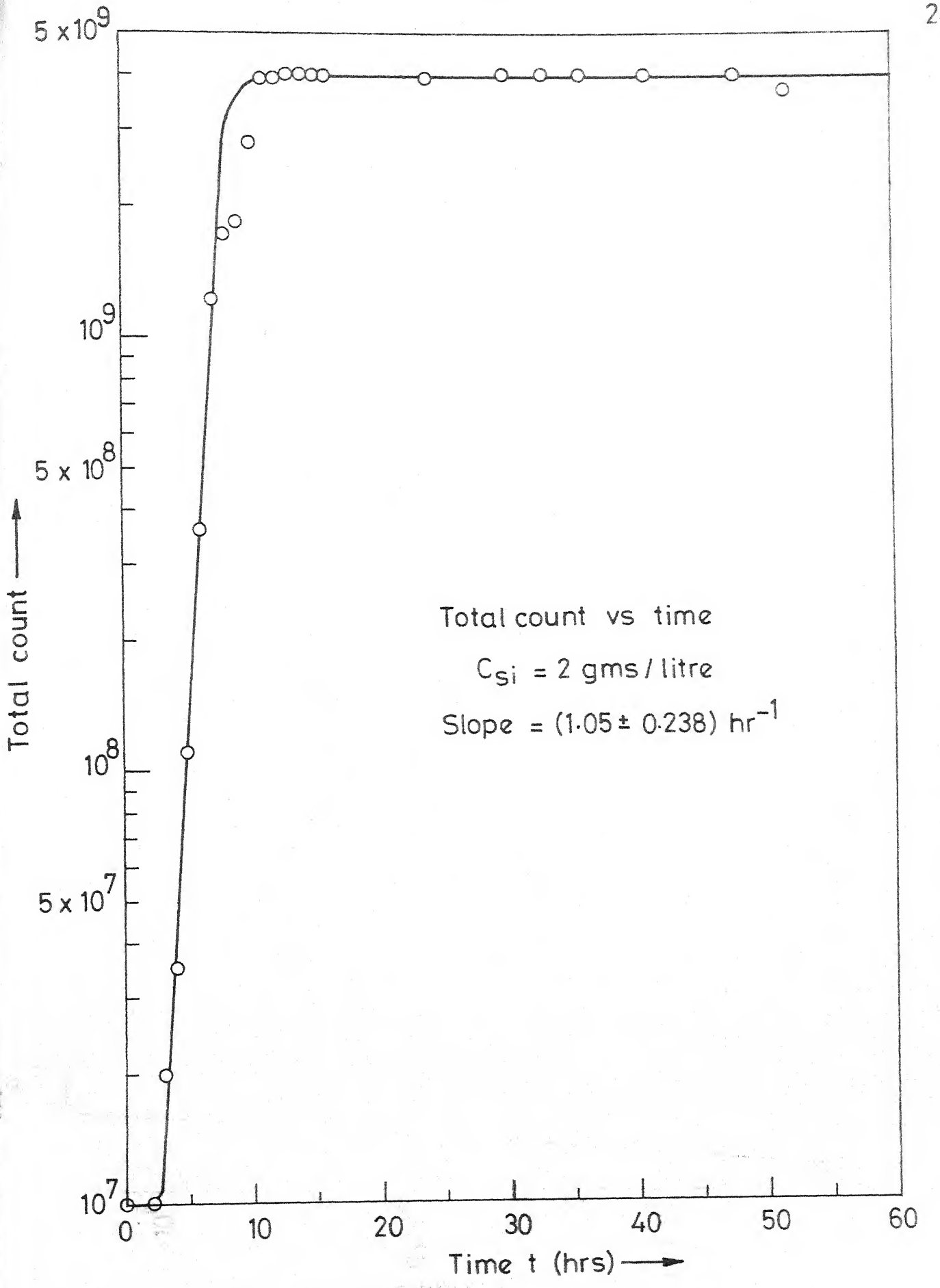


Fig. 3 - Batch culture.



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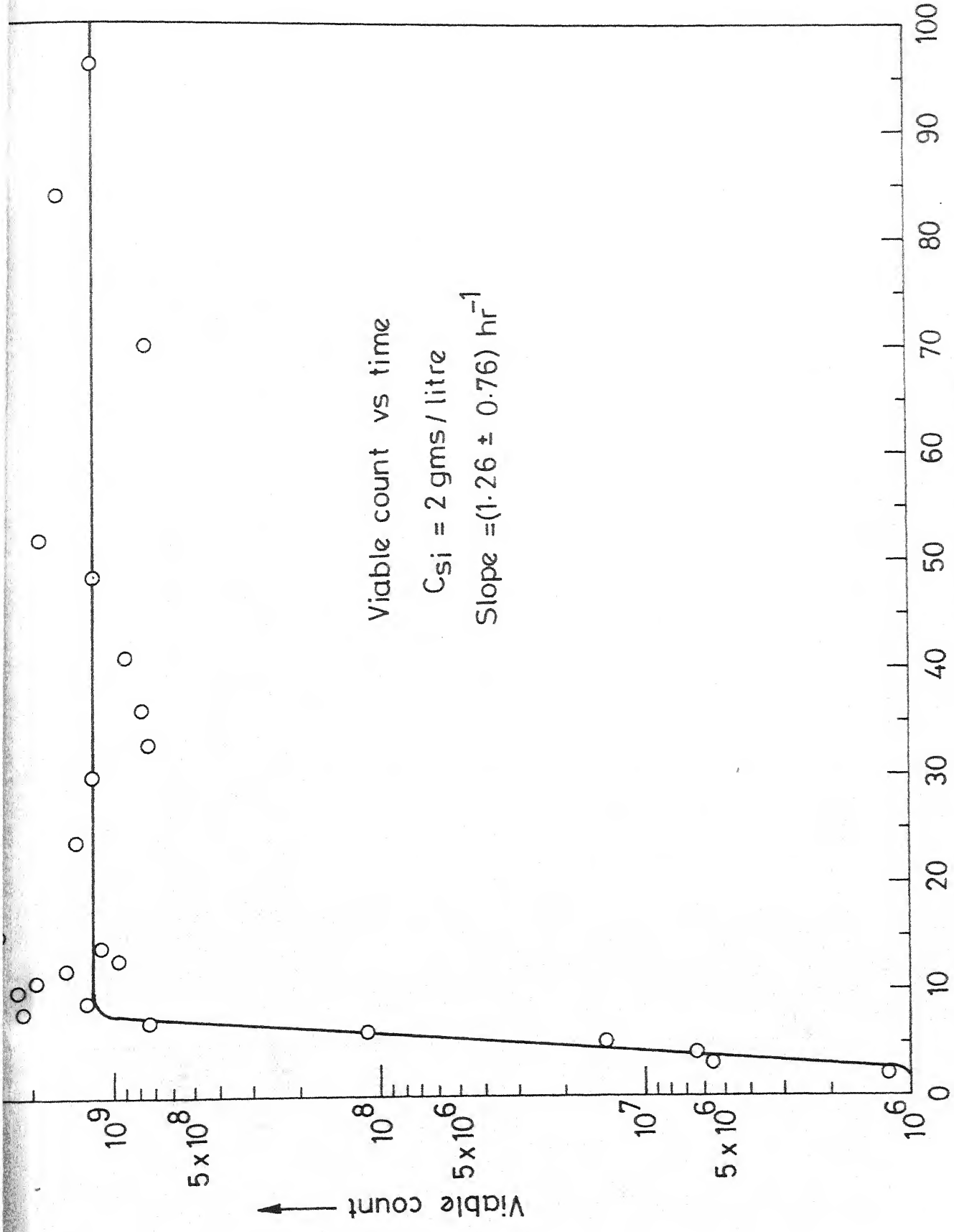

Fig.5 - Batch culture.

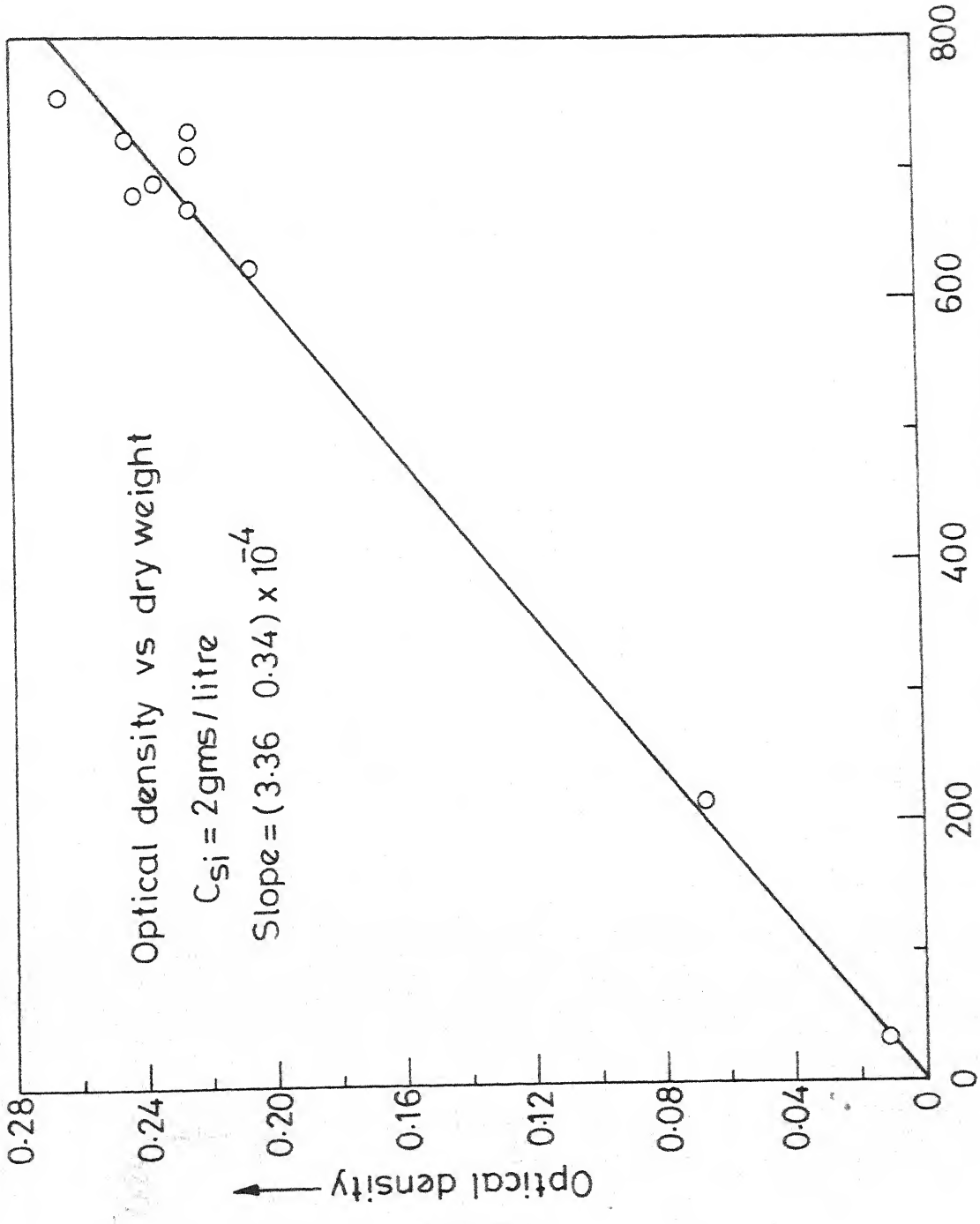

Fig.6 - Batch culture.

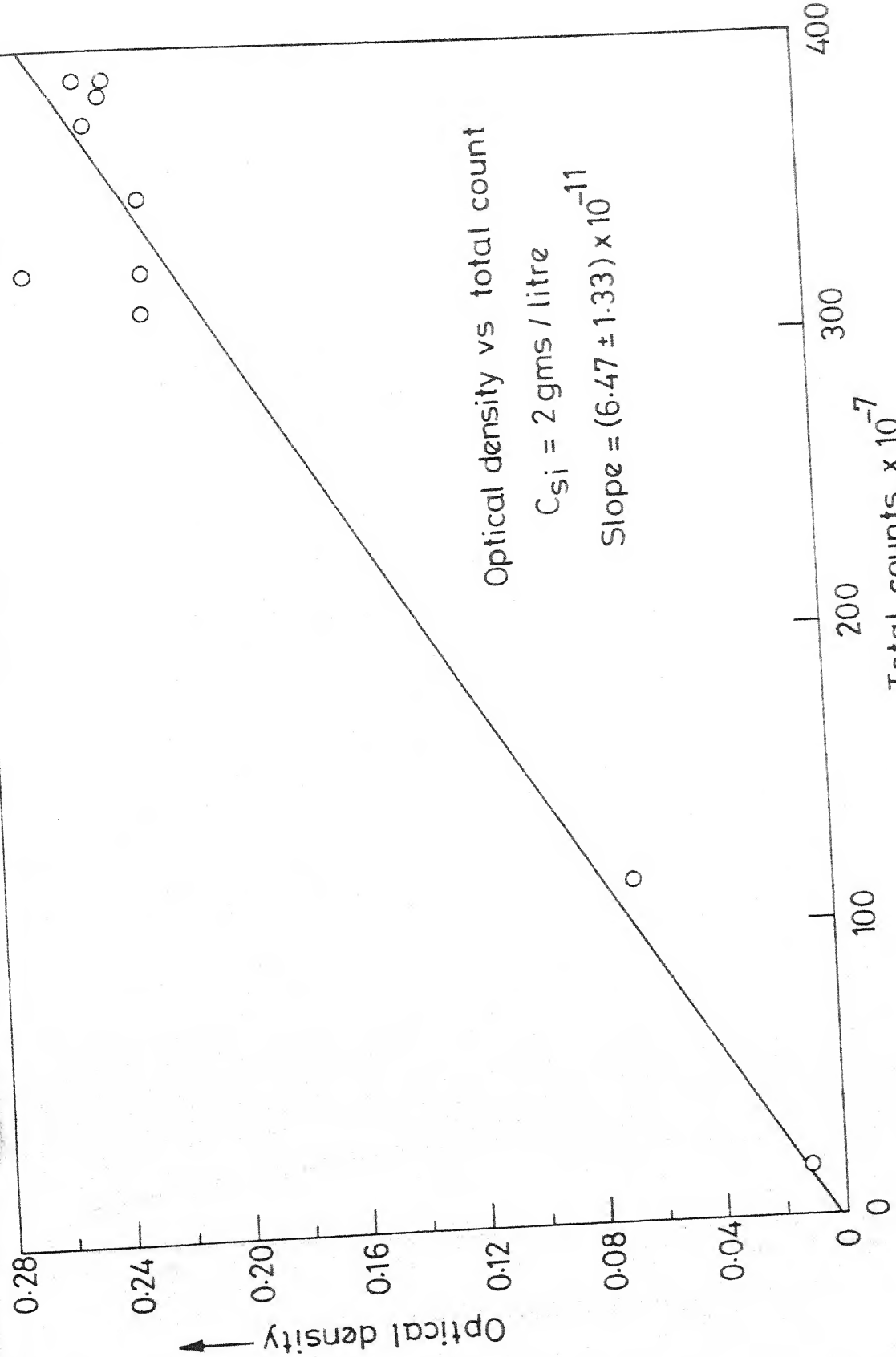

Fig. 7 - Batch culture.

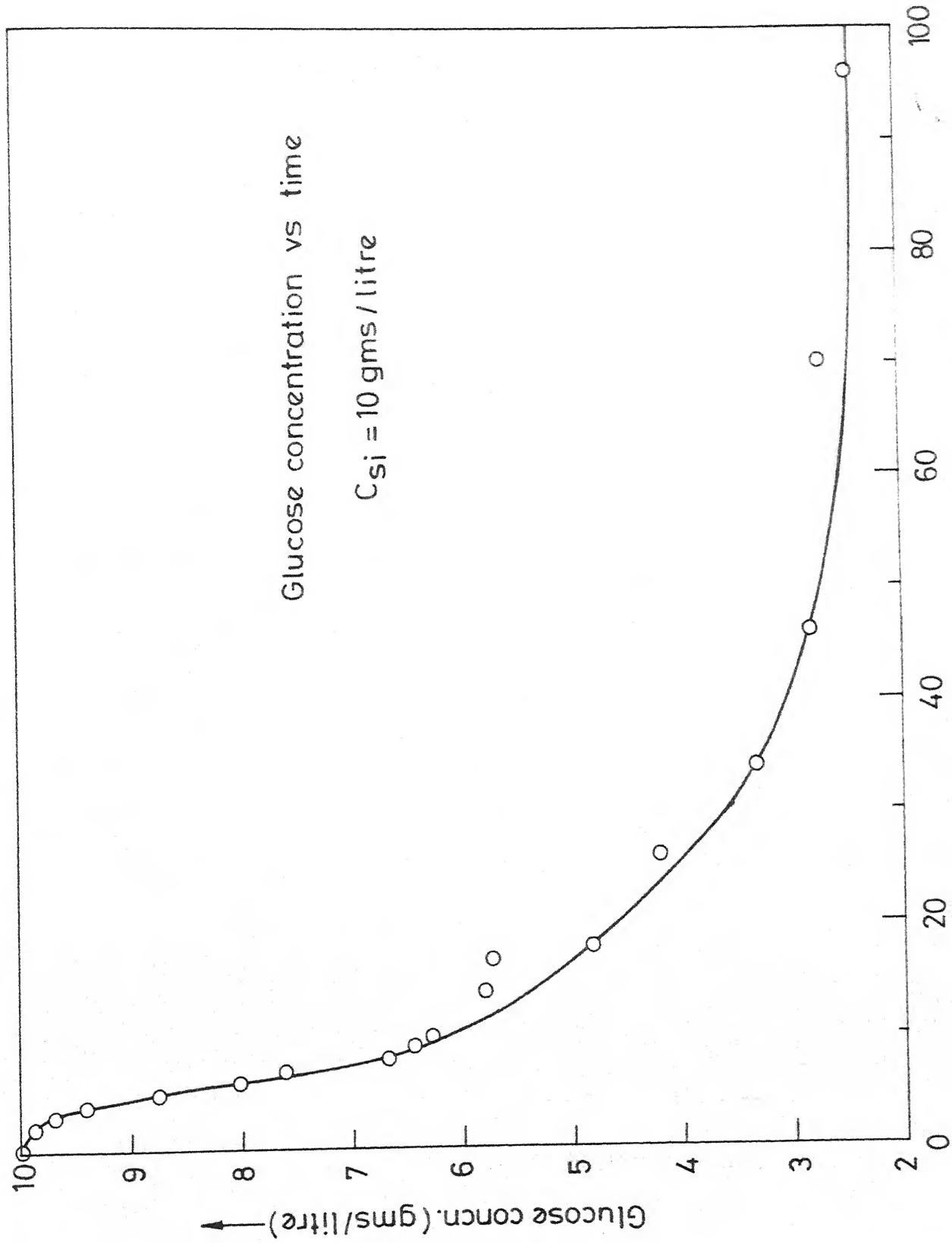

Fig. 8 - Batch culture.

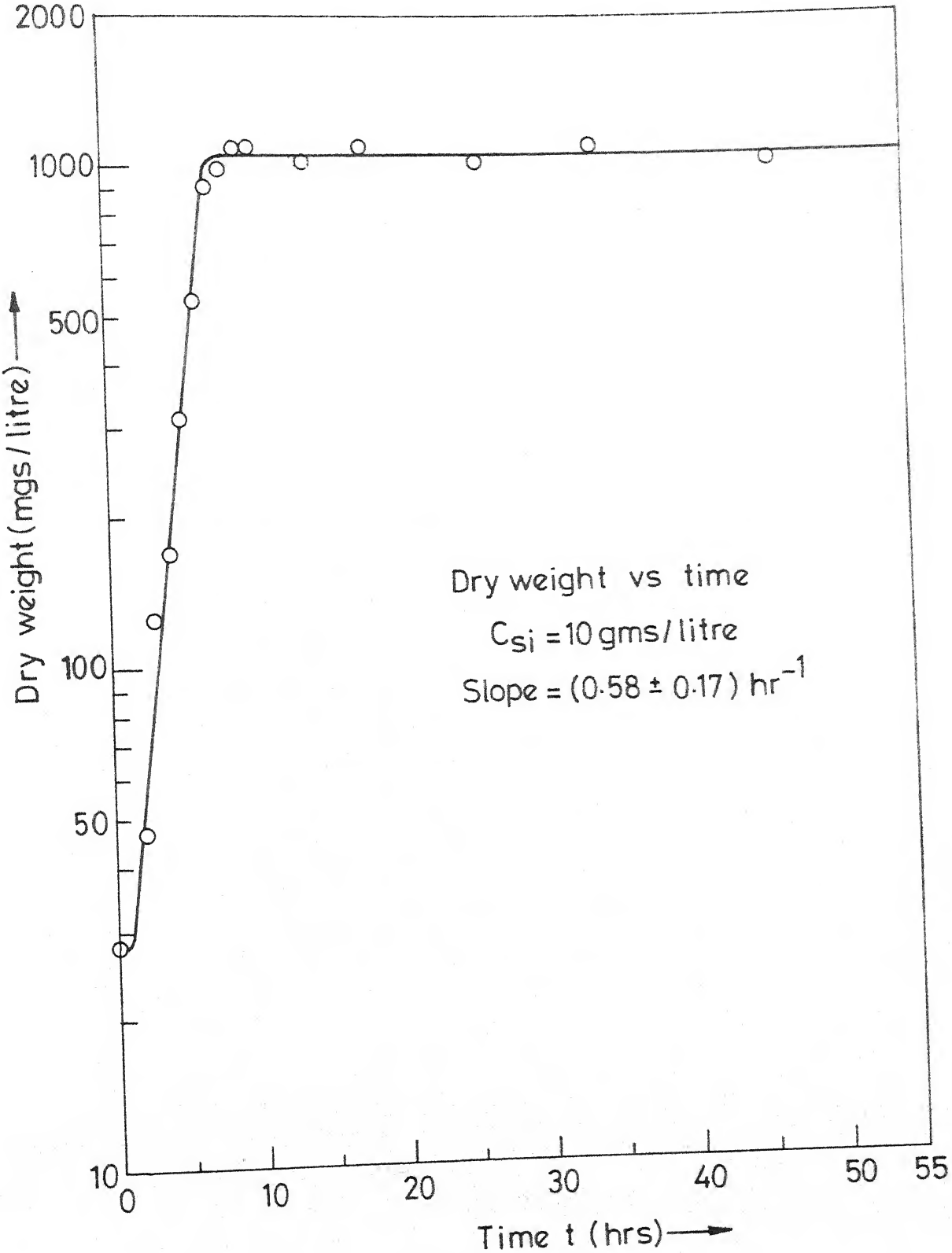

Fig.9 - Batch culture.

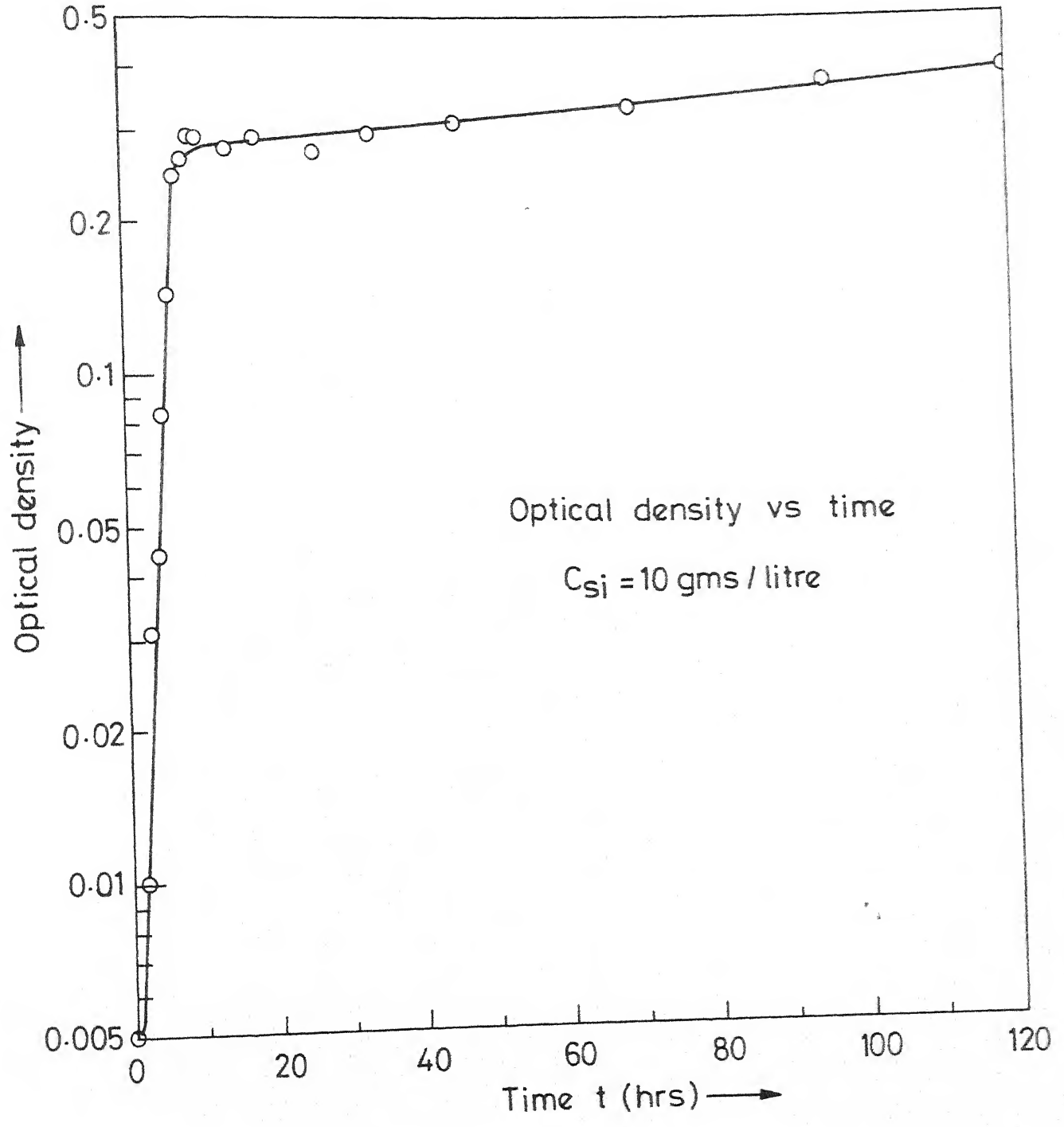

Fig.10 - Batch culture.

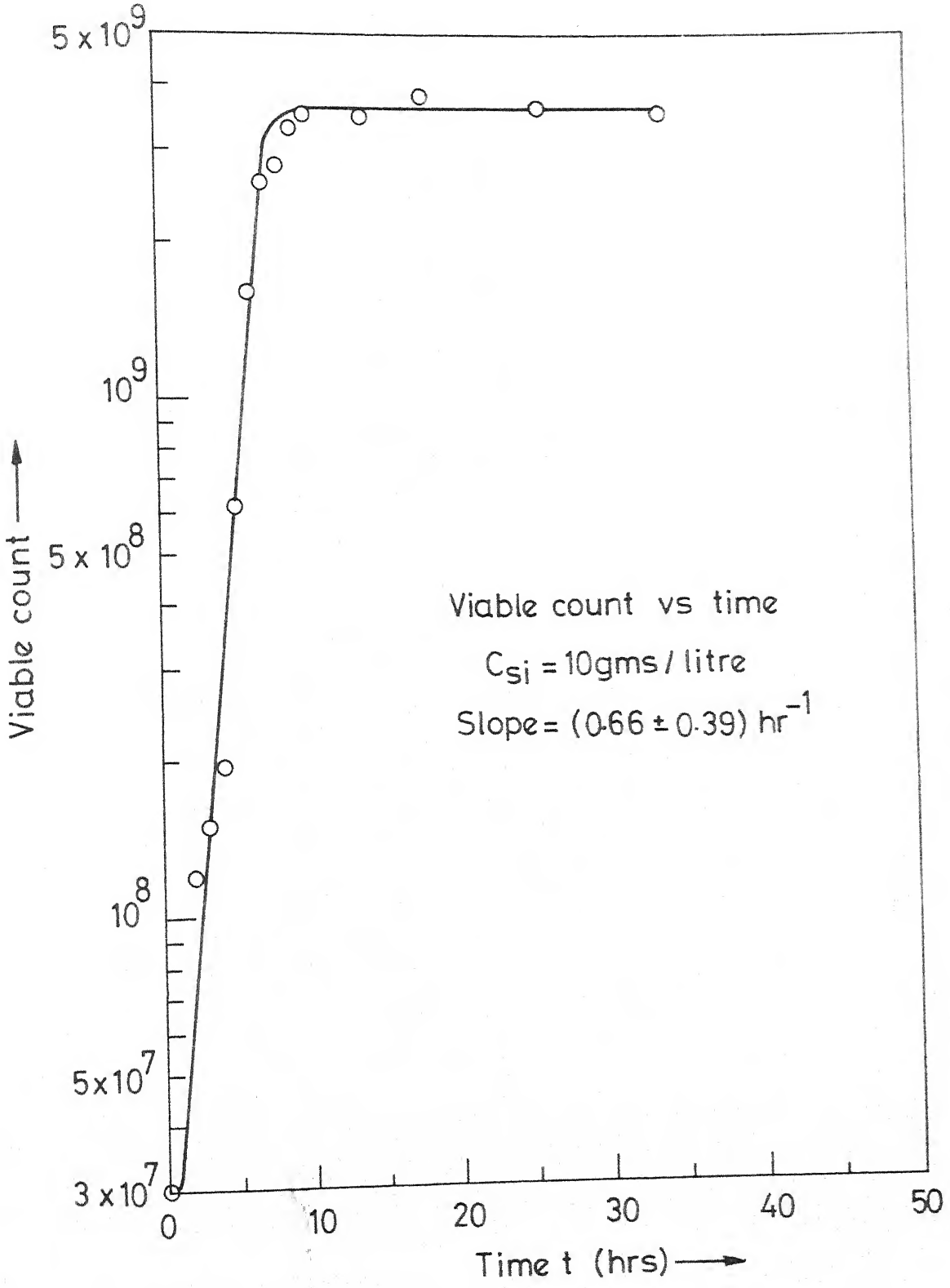

Fig.11 - Batch culture.

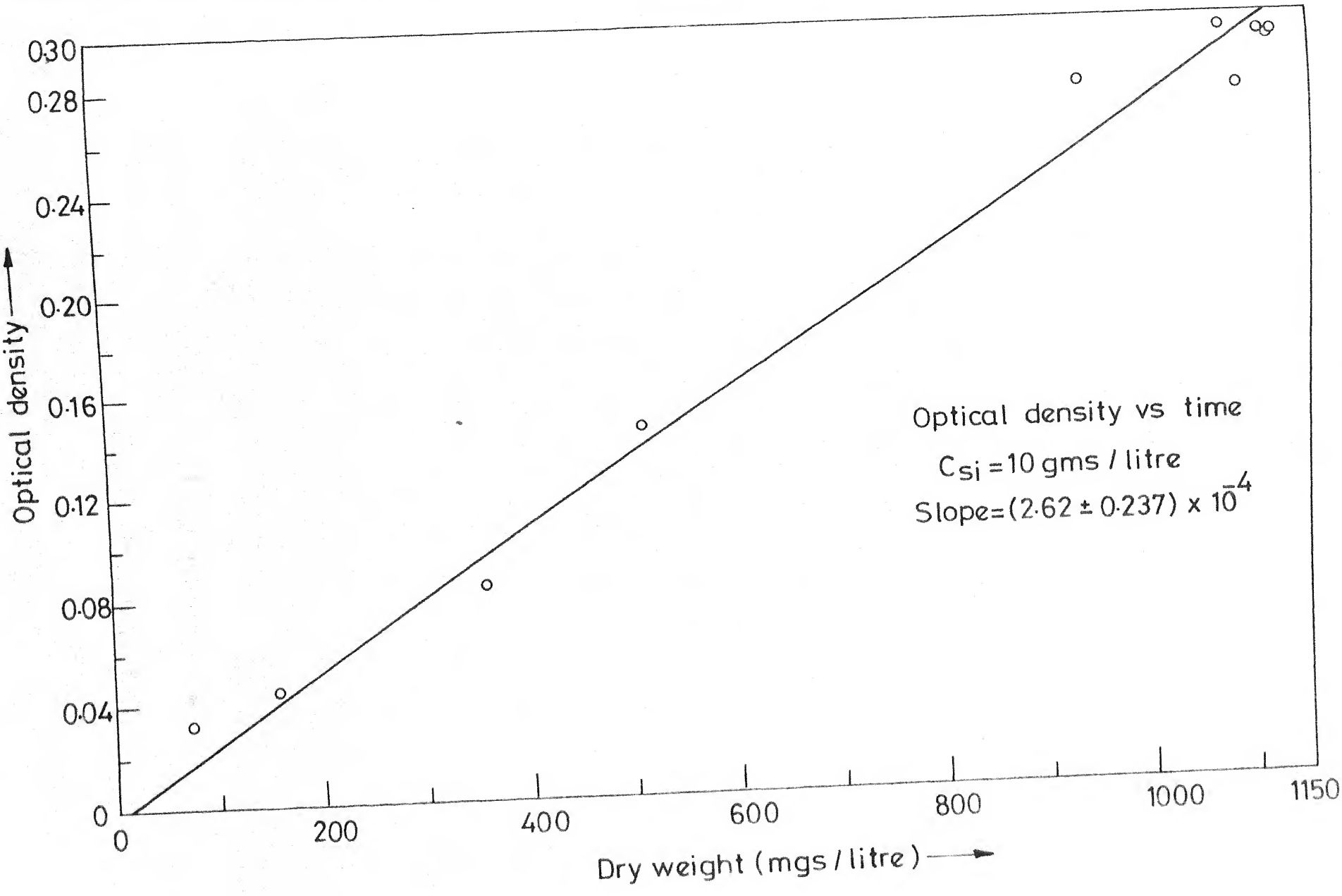

Fig.12 - Batch culture.

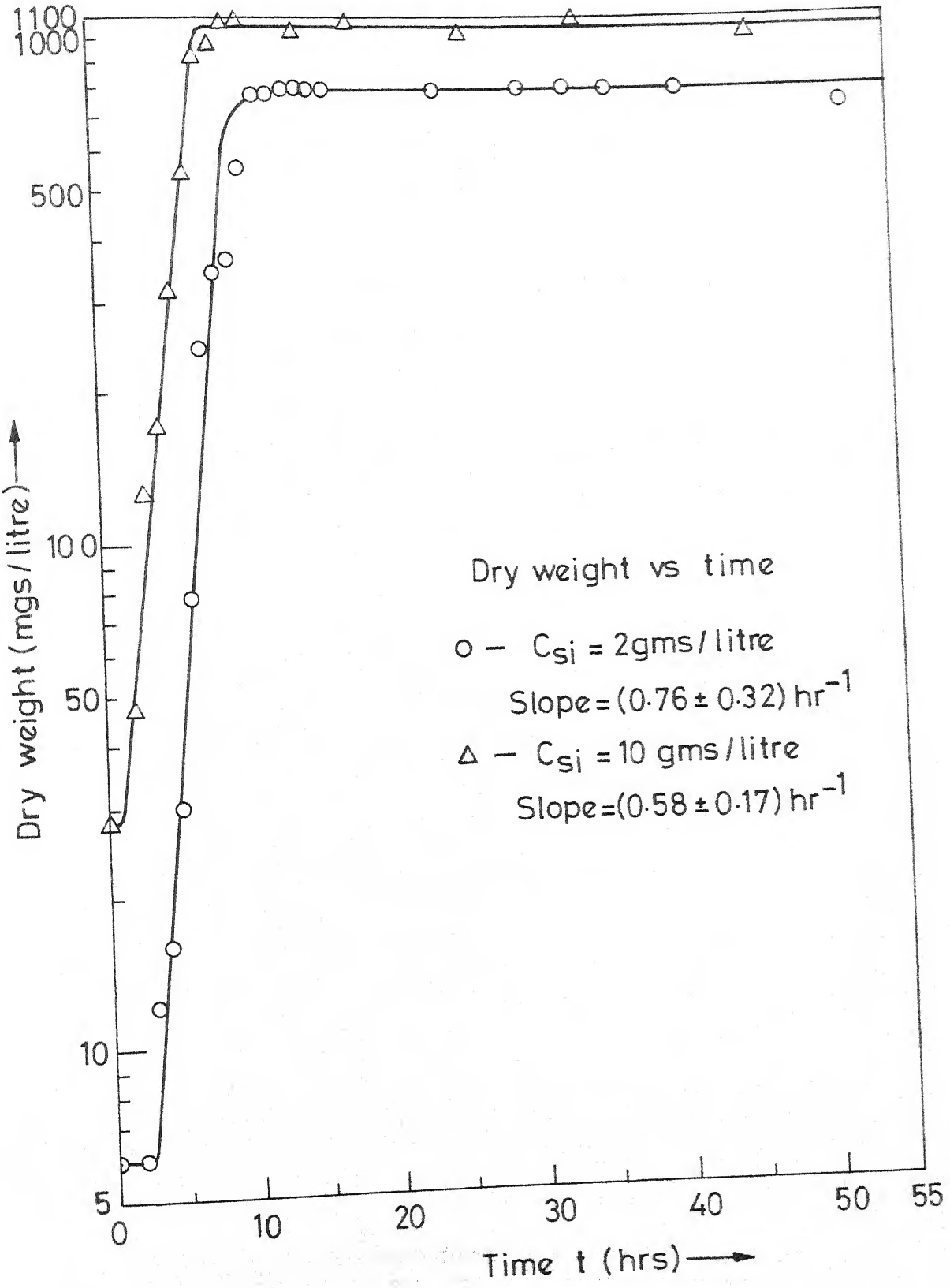

Fig.13 - Batch culture.

TABLE IV

CONTINUOUS CULTURE DATA

C_{si} = 2 gms/litre

D = 0.66 hr^{-1} (From 0 to 17 hours)

D = 0.36 hr^{-1} (From 17 to 34 hours)

Sl. No.	Time t (hrs)	Optical Density	Dry Weight C (mgms/lit.)	Viable Count x 10^{-7}	Glucose Concentration C_s (mgms/litre)
1	0	0.260	777	443	19.1
2	1	0.200	597	207	21.3
3	2	0.178	531	142	25.5
4	3	0.192	573	117	27.6
5	4	0.200	597	181	29.1
6	5	0.196	586	226	29.1
7	6	0.193	577	200	29.1
8	7	0.209	625	206	29.1
9	8	0.216	645	226	29.2
10	9	0.226	675	192	29.0
11	10	0.223	666	212	29.0
12	11	0.220	657	185	29.0
13	12	0.216	645	232	29.0
14	15	0.224	670	251	29.2
15	17	0.217	648	236	29.2
		Dilution rate changed from 0.66 hr^{-1} to 0.36 hr^{-1}			
16	18	0.232	692	293	26.65
17	20	0.244	728	230	27.10

Table -IV (Contd.)

18	21	0.246	735	283	26.65
19	23	0.245	732	233	26.65
20	26	0.234	698	223	27.50
21	28	0.228	682	267	27.10
22	31	0.230	687	265	23.80
23	34	0.232	692	247	24.20

* * *

TABLE V

CONTINUOUS CULTURE DATA

C_{si} = 10 gms/litre

D = 0.54 hr^{-1}

Sl. No.	Time t (hrs)	Optical Density	Dry Weight C (mgms/litre)	Viable Count x 10^{-6}	Glucose Concentration C_s (gms/litre)
1	0	0.30	1115	21	4.04
2	1	0.22	745	19	6.32
3	2	0.142	531	14	7.60
4	3	0.080	304	15	8.48
5	4	0.055	212	33	9.20
6	5	0.040	156	35	9.40
7	6	0.035	139	7	9.20
8	8	0.037	145	20	9.56
9	10	0.040	156	25	9.23
10	12	0.043	167	23	9.11
11	16	0.040	156	20	9.05
12	20	0.042	164	22	9.07

* * *

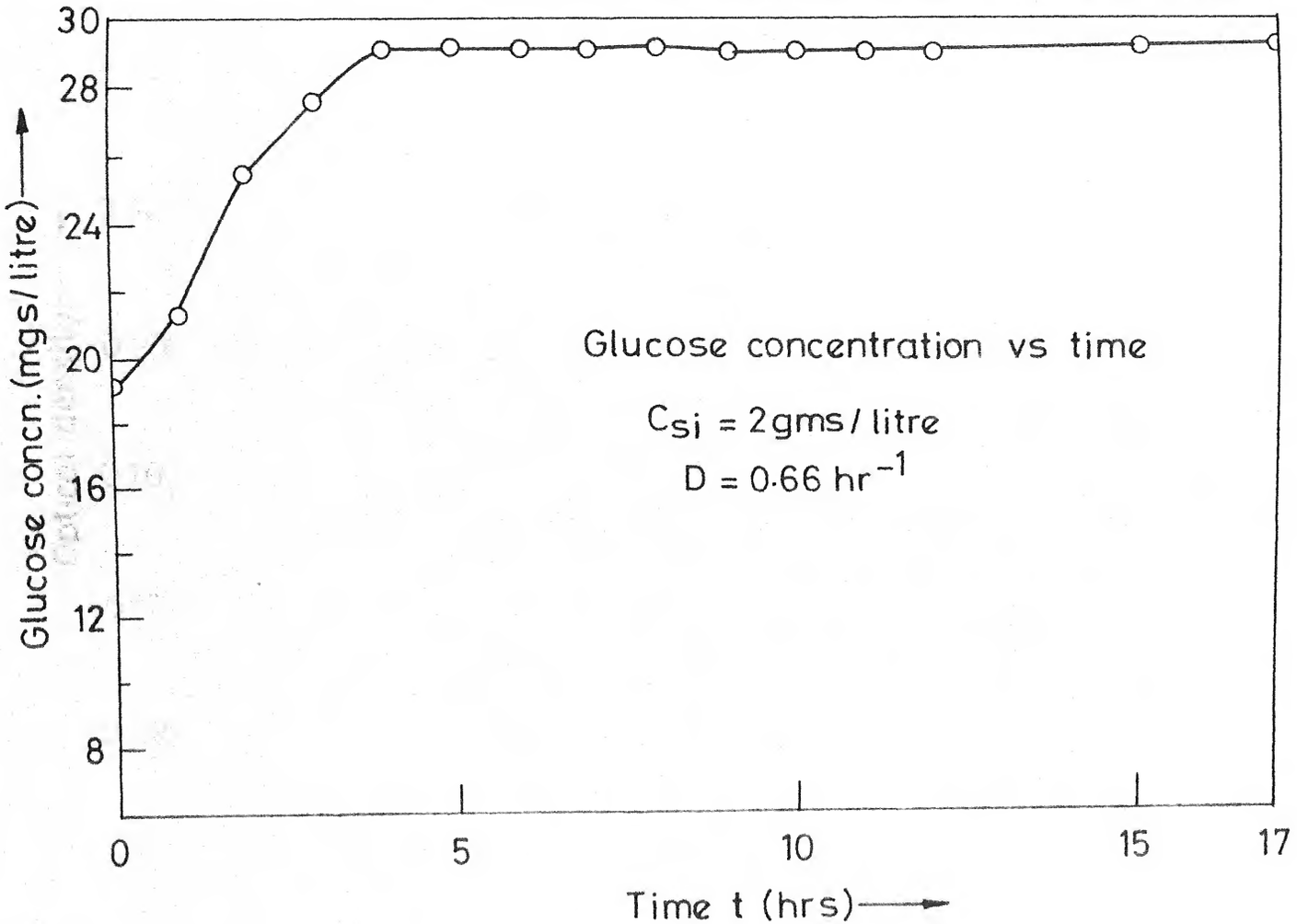

Fig. 14 - Continuous culture.

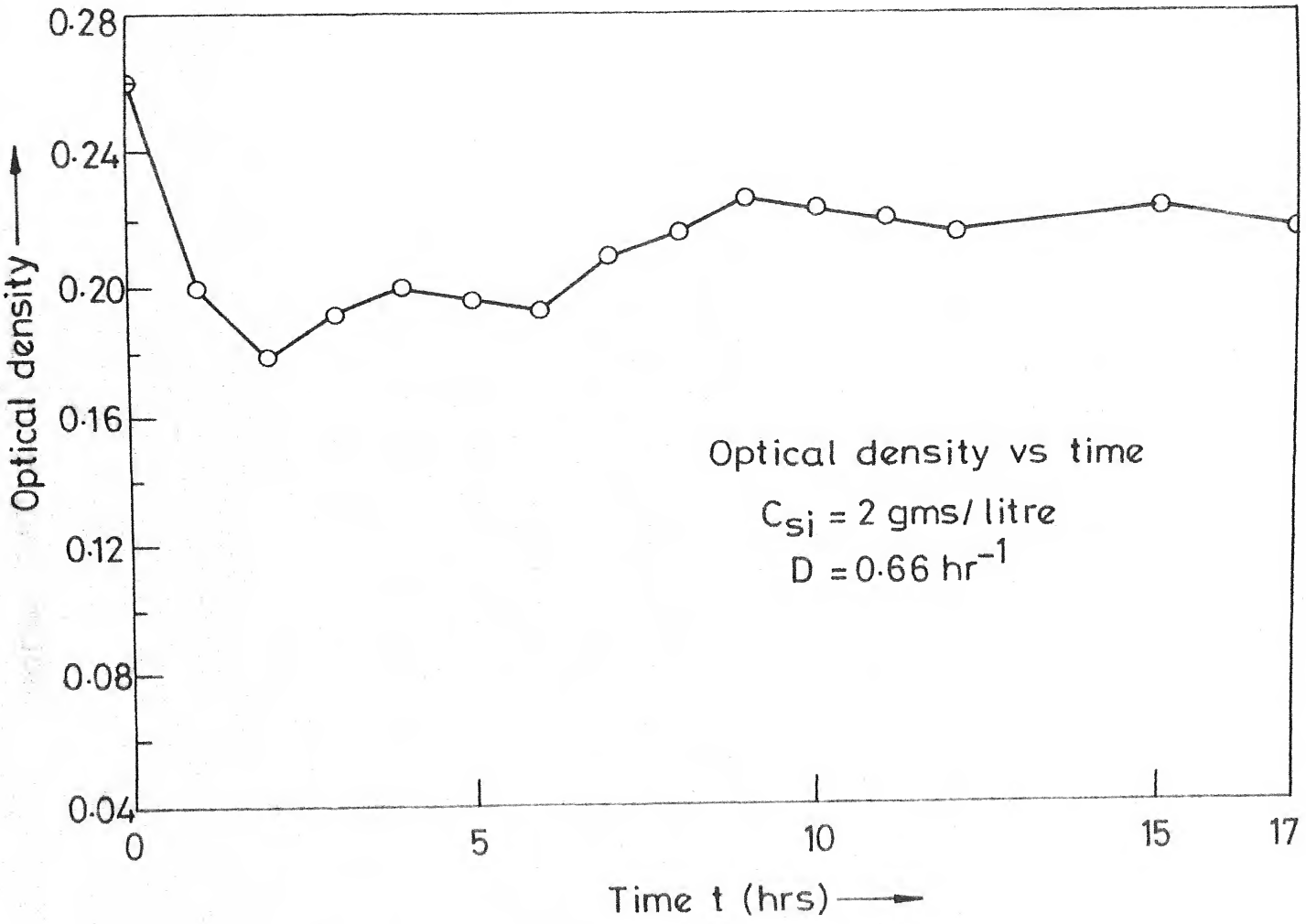

Fig. 15 - Continuous culture.

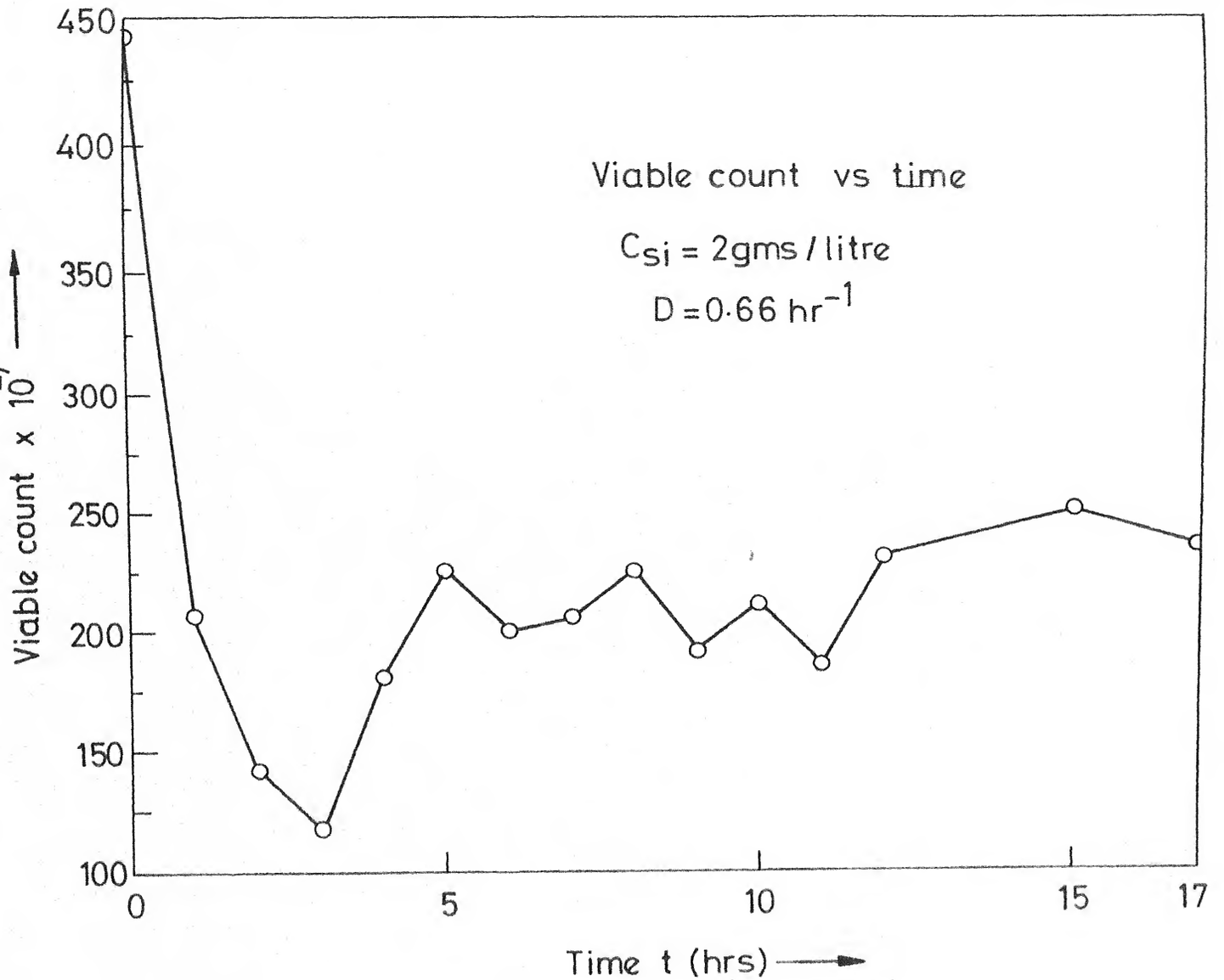

Fig.16 - Continuous culture.

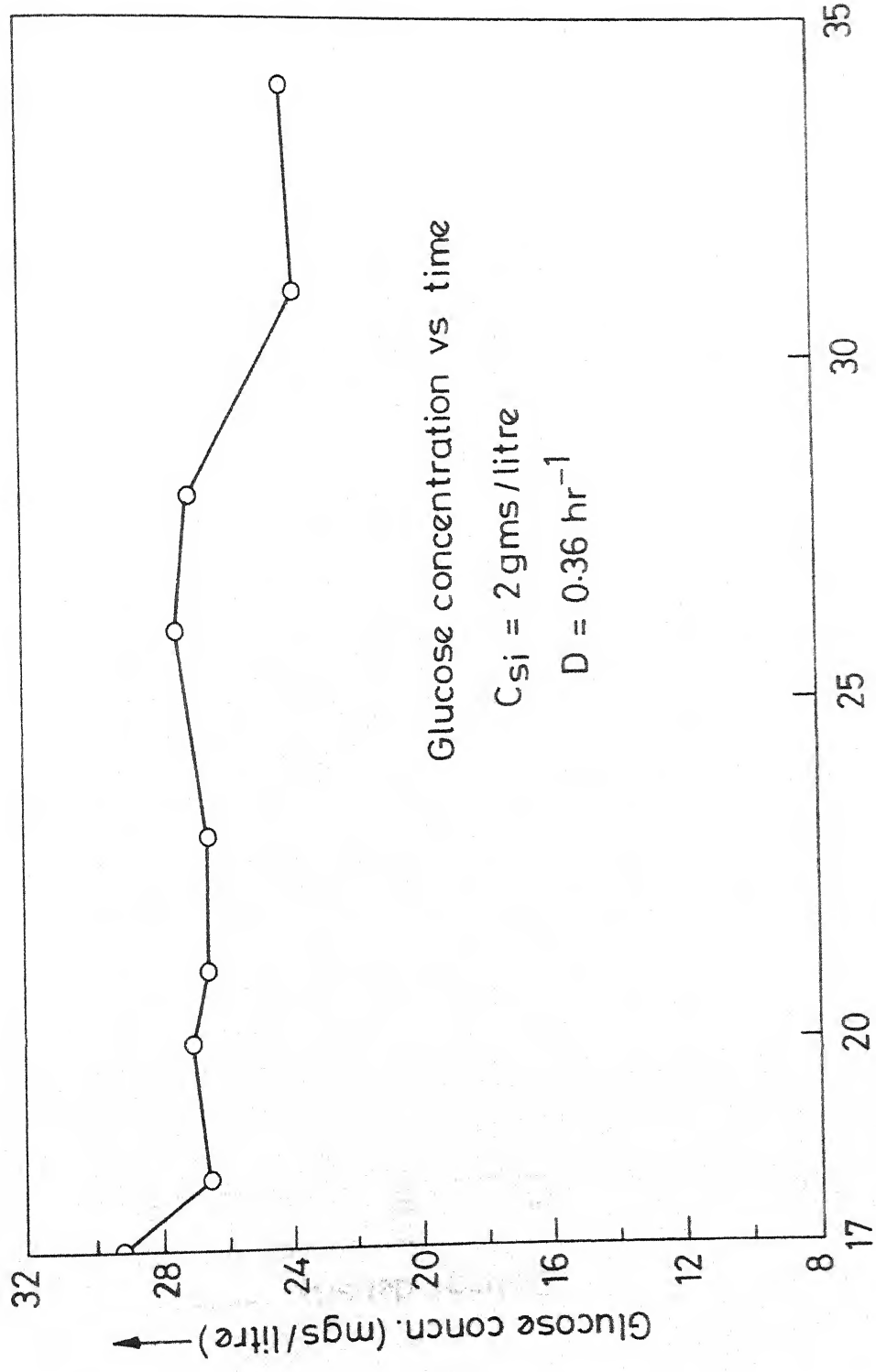

Fig.17 - Continuous culture.

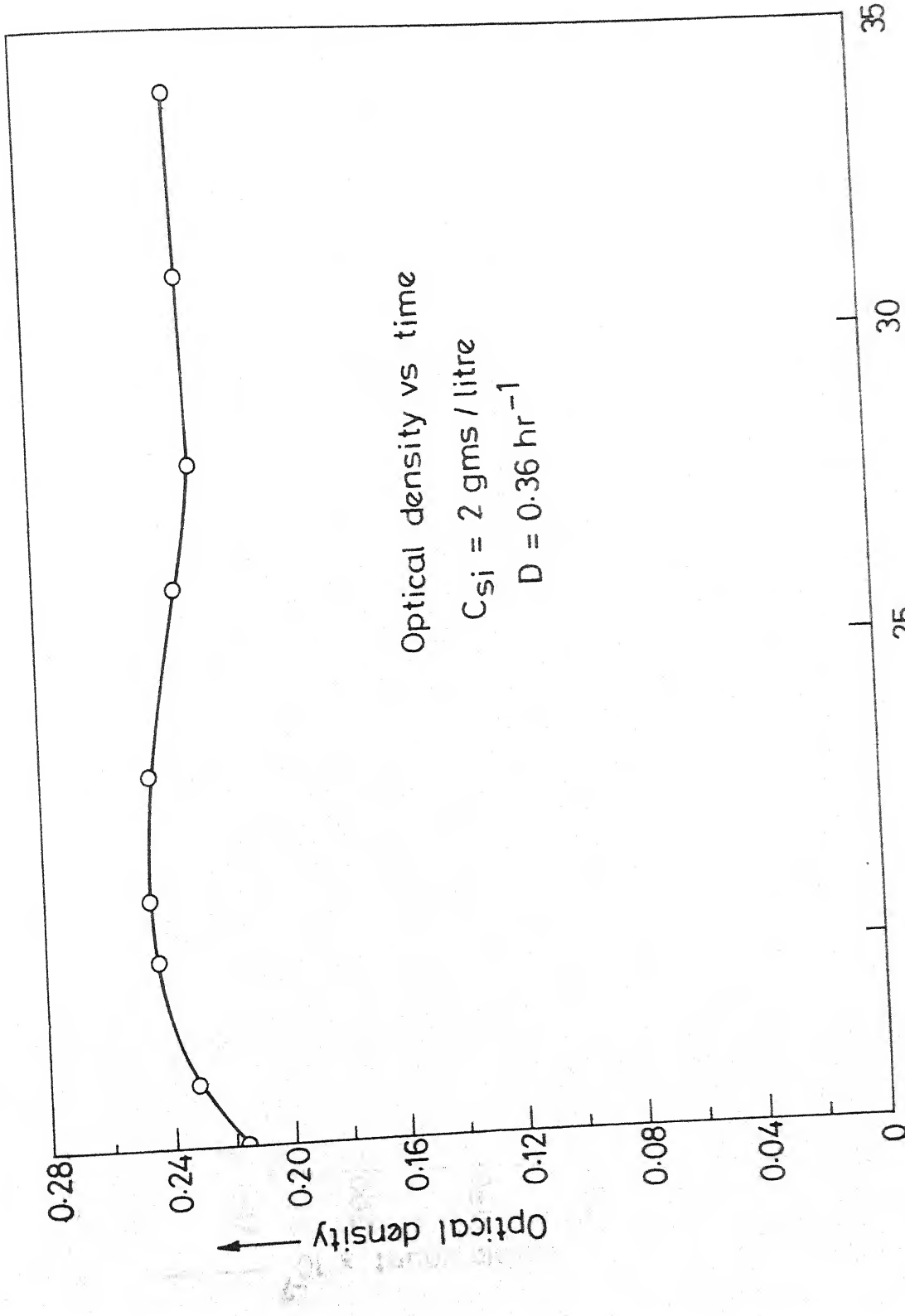

Fig. 18 - Continuous culture.

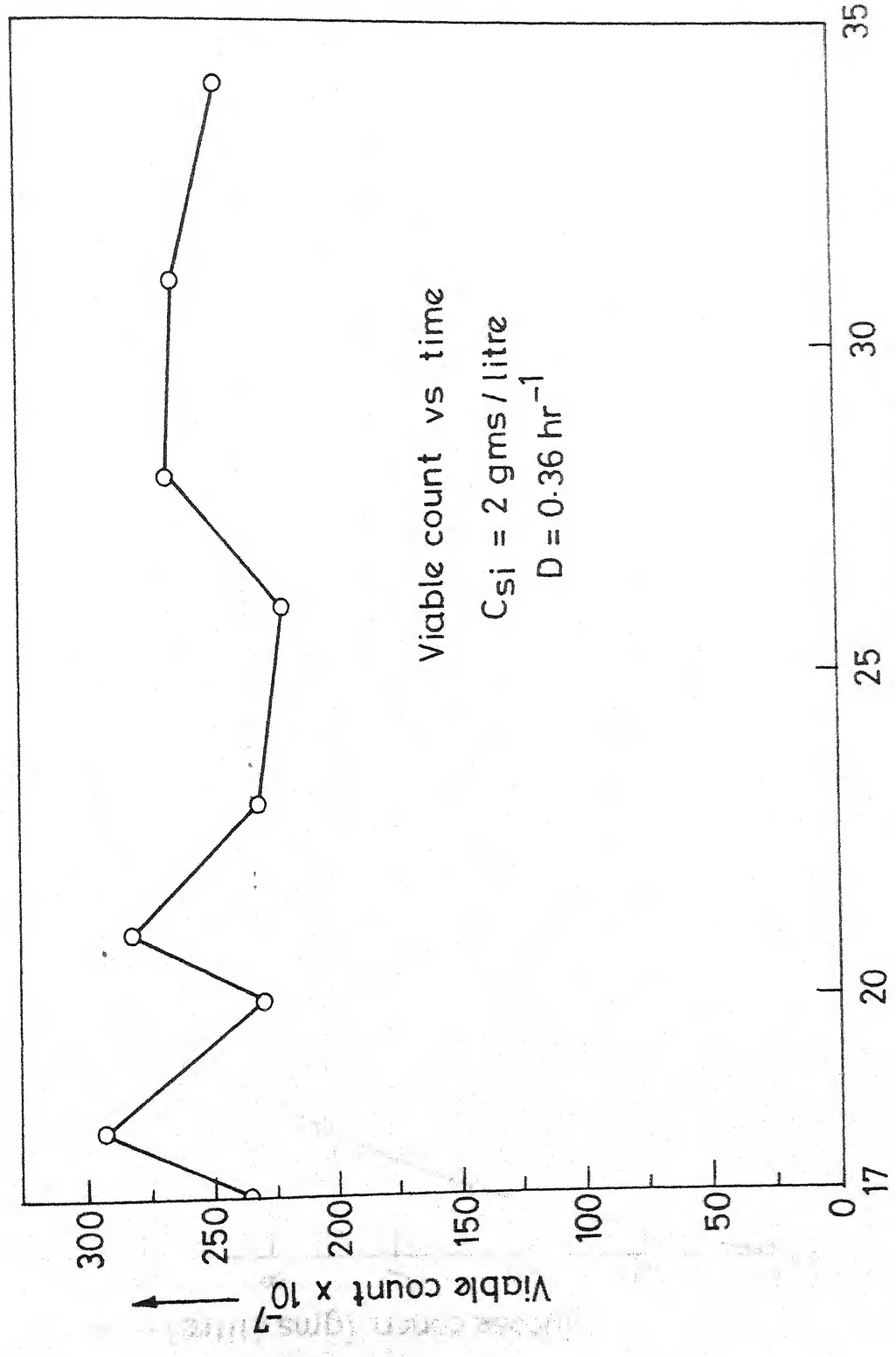

Fig.19 - Continuous culture.

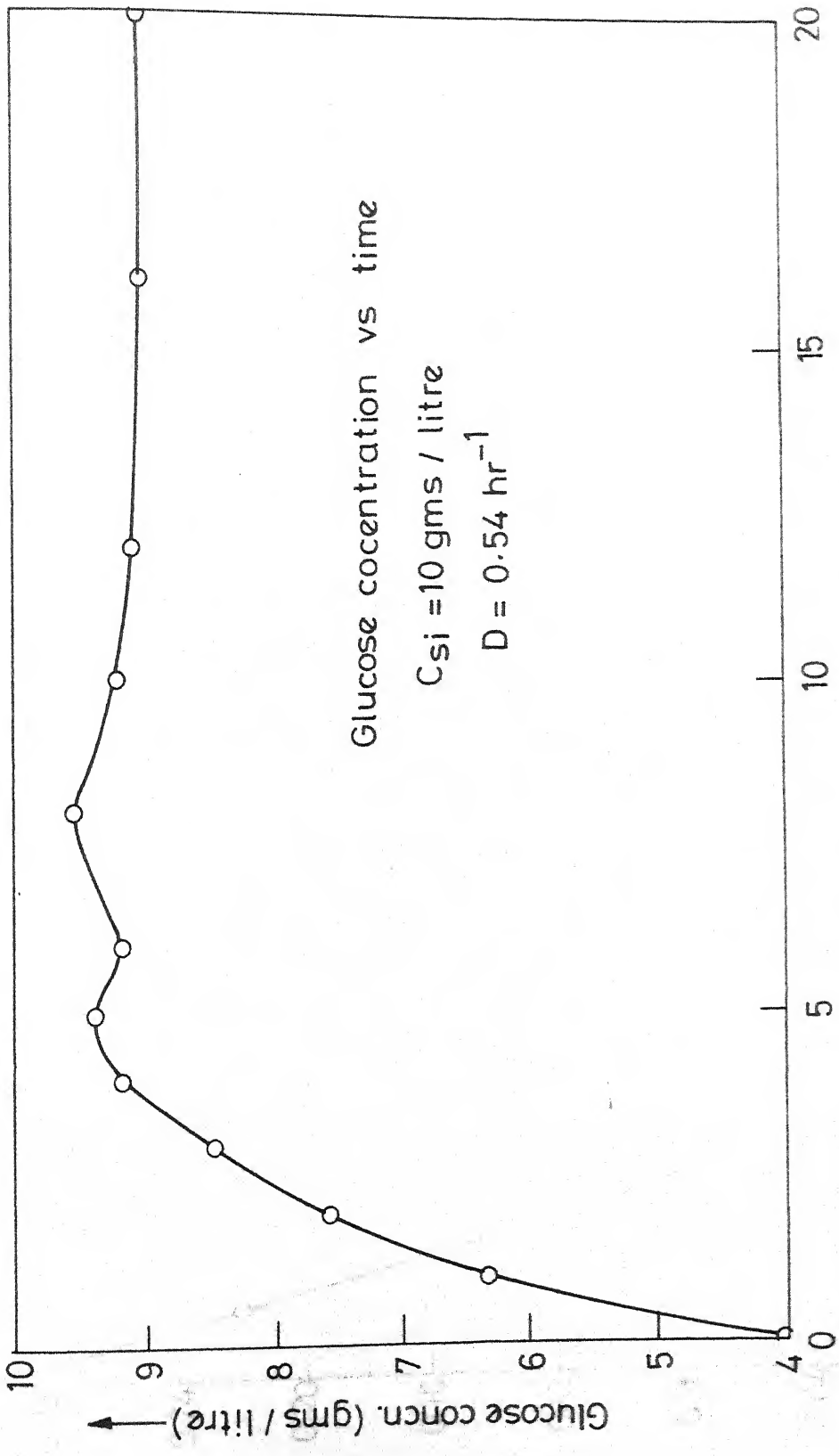

Fig. 20 – Continuous culture.

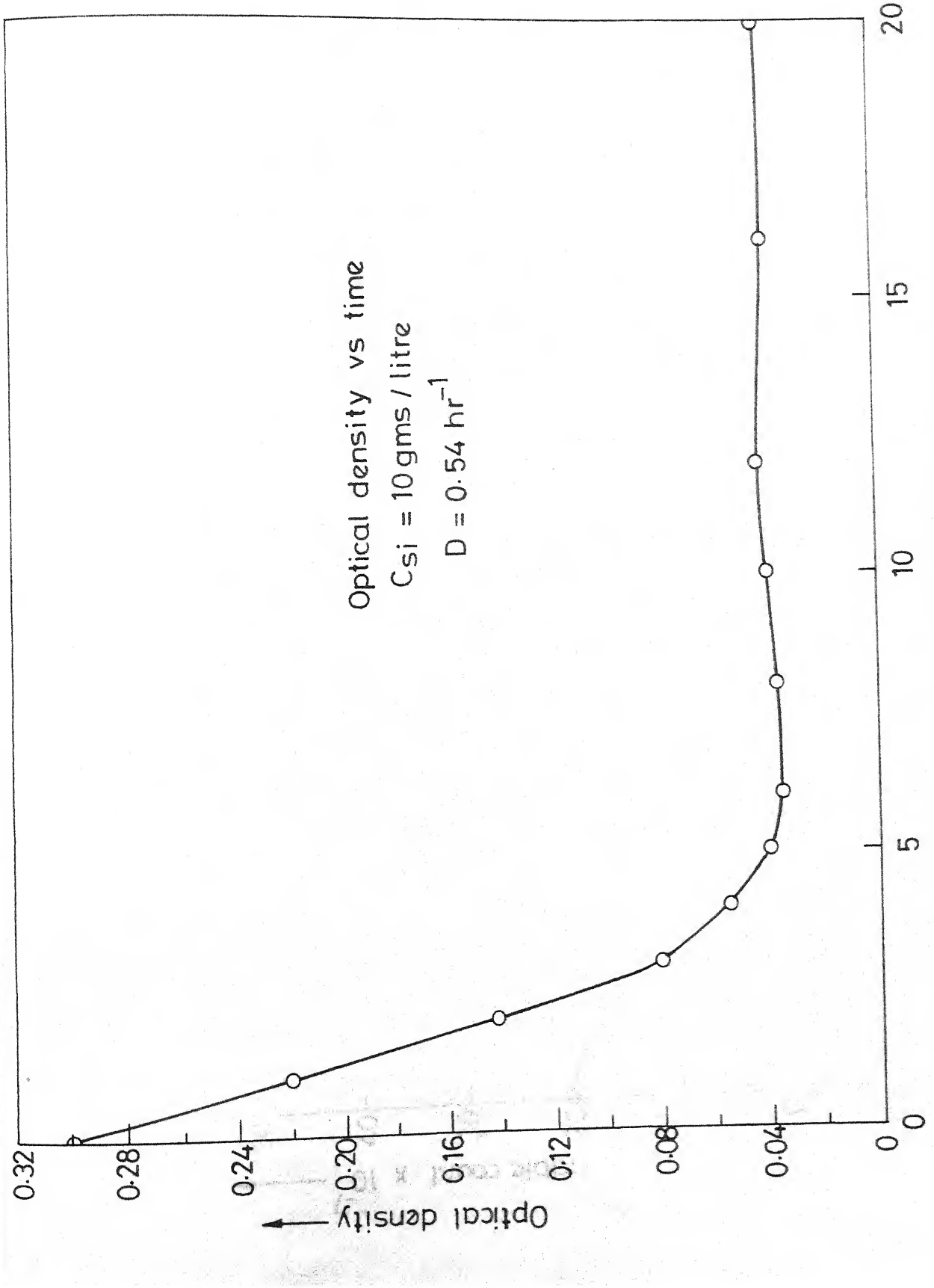

Fig. 21 - Continuous culture.

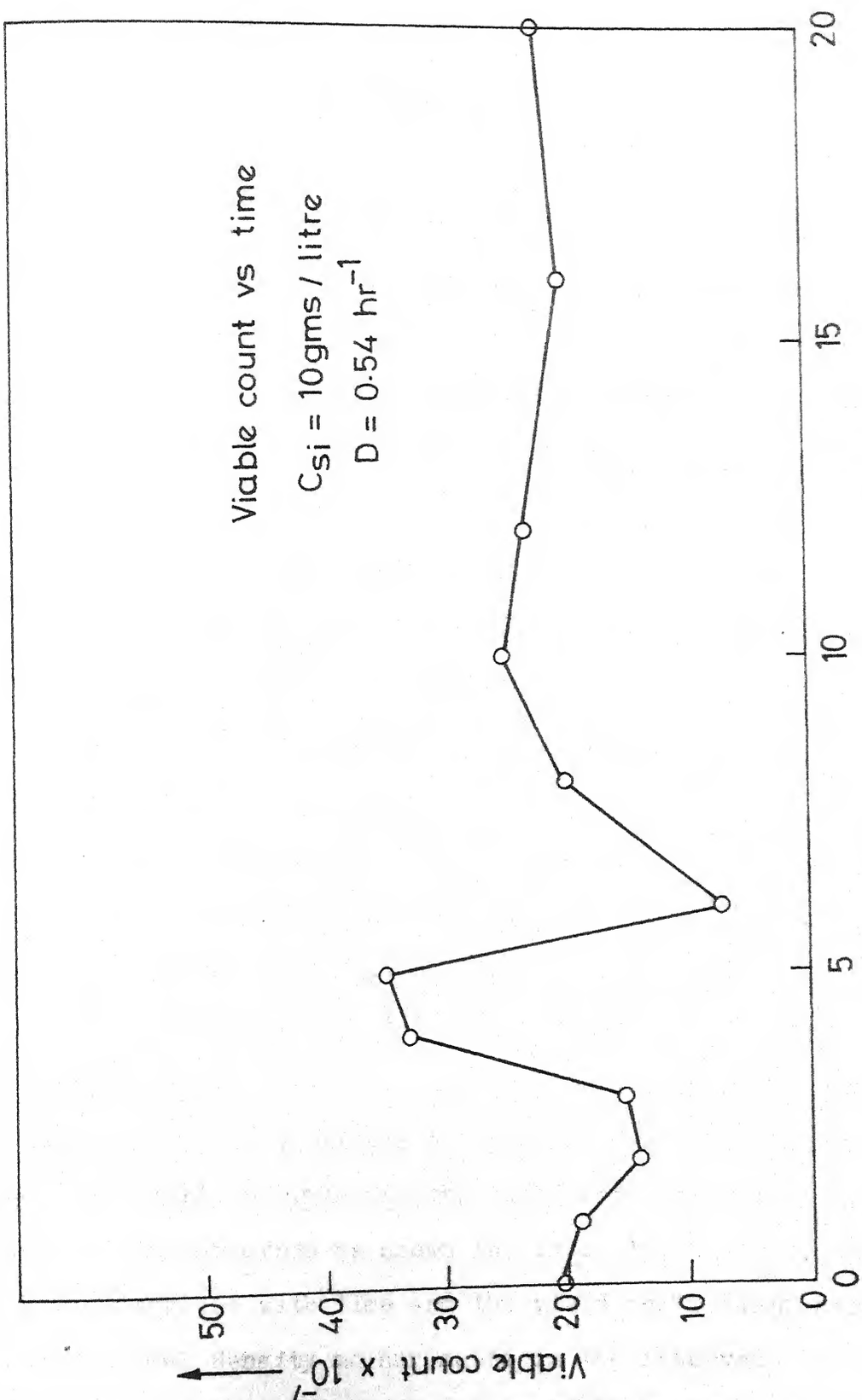

Fig. 22 - Continuous culture.

CHAPTER - IV

ANALYSIS OF BATCH AND CONTINUOUS CULTURES

Table I shows a qualitative agreement between the experimental results and those predicted from Monod's model at C_{si}= 2 gms./litre. However, at C_{si}= 10 gms./litre the Monod model, predicts results widely different from those observed experimentally.

A study of the experimental results summarized in Tables I to V and shown graphically in figures 2 to 22 show that the following results contradict Monod's model.

(i) In the batch run with C_{si}= 10 gms./litre, the glucose concentration attained a steady value of 2.42 gms./litre after about 70 hours, with no more glucose being utilized. This is shown in Figure 8.

(ii) The decrease in the value of yield coefficient (Y) in the batch run with C_{si}=10 gms./litre. This is clear from an analysis of Figures 8 and 9. In the exponential phase (between 0 to 8 hours) the yield coefficient was found to be 0.321. At the end of exponential phase after about 8 hours the dry weight did not show any increase, whereas the glucose was still being consumed in appreciable amounts and the yield was nearly zero. Optical density measurements as shown in Figure 10, however, showed a slight increase with time and the yield coefficient calculated from optical density measurements in the intervals between 8 to 20 hours and 20 to 54 hours was found to be about 0.05.

(iii) The value of specific growth rate (μ) decreased from 0.76 hr^{-1} to 0.58 hr^{-1} as the initial glucose concentration increased from 2 gms/litre to 10 gms./litre. The slope of ln C versus time plot as shown in Figures 3 and 9 was determined by the method of least-squares. The values of 95 percent confidence limits are given together with the estimated values of μ. The decrease in the value of μ from 0.76 hr^{-1} to 0.58 hr^{-1}, however, is masked by large values of confidence limits. More experimental results are necessary to verify this point.

The above results show that Monod's model does not hold good in the entire range of growth. Moreover, Monod's model predicts utilization of all of the substrate for conversion into bacterial mass. Figure 8 shows that this does not occur in the experiment.

A qualitative explanation of these results in the light of other kinetic mathematical models is presented below:

(i) An important result observed in the batch growth curve with C_{si}= 10 gms./litre was that the glucose concentration became steady at a value of 2.42 gms./litre, with no more glucose being utilized after about 70 hours. This may be due to the formation of certain inhibitory products during growth which stop the growth process, and hence the glucose remains unutilized. Kinetic mathematical models considering the effect of inhibitors have been proposed by Ramkrishna et.al./6/ and they do predict the above behaviour. The mechanism of growth suggested by them is as follows:

$$V + a_S\, S \longrightarrow 2V + a_T\, T + \ldots\ldots \qquad (1)$$

$$V + T \longrightarrow N + (1 + a_{T1})T + \ldots\ldots \qquad (2)$$

where V = active biomass or viable mass = dry weight x fraction viability,

T = inhibitor,

N = dead protoplasmic mass, and

S = substrate

The dots represent other products of metabolism. The quantities a_S, a_T and a_{T1} are stoichiometric constants. The constant a_S represents the amount of substrate consumed in the process of forming unit mass of active biomass. The constant a_T represents the amount of inhibitor formed in the process of forming unit mass of fresh protoplasmic mass. While a_S and a_T are obviously positive, a_{T1} can be negative, positive or zero. They use the Monod growth expression for reaction (1) and take the interaction between the viable mass and the inhibitor as a second order process. The differential equations for batch growth curve are

$$\frac{dC_V}{dt} = \frac{\mu_m C_S C_V}{(K_S + C_S)} - K C_T C_V \qquad (3)$$

$$\frac{dC_S}{dt} = -\frac{a_S \mu_m C_S C_V}{(K_S + C_S)} \qquad (4)$$

$$\frac{dC_T}{dt} = a_T \frac{\mu_m C_S C_V}{(K_S + C_S)} + K a_{T1} C_T C_V \qquad (5)$$

The constants μ_m and K_S are the constants in the Monod model. and K is the rate constant for the deactivation process.

the reciprocal of the yield coefficient Y of Herbert et.al./4/. The difference between Y and $1/a_s$ will be discussed while explaining the decrease in the value of yield coefficient observed in the batch run at higher glucose concentration and in continuous culture runs. The continuous culture equations can be obtained by a simple extension of the batch growth equations. These models predict that growth can come to an end as a result of acumulated inhibitory products and the substrate need not be completely utilized.

(ii) The decrease in yield coefficient(Y) could be explained in two ways, based either on the formation of inhibitory products during growth/6/ or on the substrate consumed to maintain the viability of the cells /7/. The two possibilities are discussed below:

In the discussion of point (i) earlier, it was pointed out that the constant a_s as used in the models proposed by Ramkrishna et.al./6/ is not the same as the reciprocal of yield coefficient (Y). If there were no deactivation of the viable mass, then Y should be the reciprocal of a_s. However, when the deactivation process is present, a_s and 1/Y would differ. Thus, a_s represents the amount of substrate consumed in the formation of unit mass of protoplasmic mass without accounting for the deactivation process. The deactivation process, if present, would reduce the net gain of protoplasmic mass by growth and hence the yield coefficient (Y) would decrease. This might explain the progressive decrease in the value of Y with time in the batch run with C_{si} = 10 gms./litre because the inhibitor concentration will increase with increasing organism concentration. This might also explain the decrease in yield coefficient in the batch run with C_{si}= 10 gms./litre compared to that with C_{si}=2 gms./lit.

as shown in Table I. The continuous culture studies of the inhibitor models proposed by the above authors also predict the decrease in the value of Y with increasing holding times. This is because with increasing holding time the inhibitor concentration also increases. This could explain the decrease in yield observed in the continuous process as compared to the batch process, as shown in Table I.

The other mechanism by which the decrease in yield could be explained is based on the phenomenon of endogenous metabolism. Again, the dynamic behaviour of a microbial propogator considering endogenous metabolism has been studied by Ramkrishna et.al.[7]. They suggest the following mechanism for growth:

$$V + a_s S \longrightarrow 2V + \alpha P \qquad (6)$$

where P is an endogenous substrate formed as a byproduct of growth, α is the stoichiometric constant, Both, endogenous and exogeneous substrates are utilized to maintain the viability of cells during the entire growth period. They have proposed the following interactions.

$$V \xrightarrow{k_1} V' \xrightarrow{k_2} N \qquad (7)$$

$$V' + b_s S \xrightarrow{k_3} V \qquad (8)$$

$$V' + a_p P \xrightarrow{k_4} V \qquad (9)$$

where V' is the degradation product which can be converted to viable mass and N is the dead or nonviable mass. k_1, k_2, k_3 and k_4 are rate constants. By making a stationary state hypothesis, the concentration of V' is eliminated as a variable in the model. They have then carried out complete analysis of the batch and

continuous cultures after writing the appropriate differential equations for the two systems which are straight forward. This model also predicts a decrease in the yield coefficient with increasing time in a batch culture or with increasing holding time in a continuous culture. This is because with increasing bacterial concentration increasing amounts of the exogenous and endogenous substrates are utilized for providing the energy to keep the cells viable.

(iii) Yano and Koga [8] and Andrews [9] have shown that the growth limiting substrate may become inhibitory at higher concentrations and have proposed the following expression for the specific growth rate (μ) which is similar to the substrate inhibition kinetic expression for enzyme reactions.

$$\mu = \frac{\mu_m}{K_s/C_s + 1 + \sum_{j=1}^{n} (C_s/K_j)^n} \qquad (10)$$

where K_j's are constants.

The following observation was made about the effect of glucose concentration on the nature of bacterial colonies during the experiments. The viable count in the batch run with 2 gms/litre glucose concentration attained a steady value at the end of exponential phase. The colonies observed did not show any change in their characteristics and also the viable number was nearly constant when plated even after 120 hours. However, in the batch run with 10 gms./litre glucose concentration, after about 30 hours when the exponential phase was already over, the colonies observed were completely different from the normal ones observed earlier. The colonies were found to

have lost their well defined flat disc type of appearance and they appeared in the form of specks with no definite shape. Multiplicate platings of the same dilution of a given sample showed viable counts sometimes differing by a factor of hundred or more. It was, therefore, not possible to estimate the viable count with any accuracy. It is likely that toxic products of metabolism may be responsible for bringing about the change in colony characteristics observed. However, this point needs to be investigated further.

* * *

CHAPTER - V

CONCLUSIONS AND RECOMMENDATIONS

A. Conclusions

The batch and continuous culture experiments show that the Monod model is not valid in the entire range of growth and substrate concentration. Other kinetic mathematical models as those proposed by Ramkrishna et.al. considering the formation of inhibitory products of metabolism during growth and the phenomenon of endogenous metabolism, do provide a qualitative explanation of the results obtained. The data collected was insufficient for a rigorous test of these models. A large number of batch and continuous cultures experiments over a wide range of limiting substrate concentration and dilution rates, will have to be performed to test the validity of these models.

B. Recommendations

Some of the difficulties experienced during the course of this work have been summarized in the following. Possible modifications to overcome them have been suggested.

(i) The continuous culture vessel should be provided with internal baffles and antifoaming agents should be added to the liquid culture so as to destroy the undesirable foam which starts forming after the experiment has progressed for few hours. The foam contaminates the inlet of the nutrient medium feed line to the cultivator.

(ii) Silicon rubber tubing should be used wherever a tube connection is necessary. This is durable and is not affected by

sterilization temperature and pressure. Ordinary rubber tubing cannot withstand the high sterilization temperature and pressure for long period of time. It becomes useless after a single continuous run and has to be replaced. Use of rubber tubing in nutrient medium feed lines may also be harmful for the growth of microorganisms because of the possibility of toxic products from rubber entering the liquid medium.

(iii) During the experiments, the temperature of the culture vessel was maintained constant by placing the whole set-up in a thermostatic chamber. This is not an efficient and accurate method of controlling the temperature. Independent control of the temperature of the culture vessel, by using a constant temperature water bath or water cooling coils surrounding the culture vessel, is desirable. As other parts of the continuous culture set-up are susceptible to temperature changes, the whole set up should still be kept in a thermostatic chamber. Once temperature of the culture vessel is controlled independantly, it becomes possible to operate the thermostatic chamber at much lower temperature, thus greatly improving the working conditions inside the chamber.

(iv) An ordinary hemocytometer, actually meant for blood cell counts, was used for taking total counts of the bacterial cells. It was very difficult to get accurate bacterial cell counts as there could be four to five layers of cells in the space between the cover slip and the slide. Hence,all the cells could not be counted at one focussing of the microscope. The Petroff-Hauser or Helber bacterial counting chamber specially meant for bacterial cell counts could be used to get improved results.

(v) Glucose when present in higher concentrations of 5 gms./ litre or more was found to be unstable to steam sterilization. It was, therefore, separately sterilized and added asceptically to rest of the nutrient medium. Glycerol can be used as carbon source instead of glucose as it is stable to steam sterilization.

(vi) Lastly, special mention must be made of the lack of a well-equipped microbiological laboratory. During this work appreciable time and efforts were spent in procuring or repairing some of the essential equipment needed for the experimental work. It was, therefore, not possible to collect batch and continuous culture data for a wide range of glucose concentrations and dilution rates as originally planned.

* * *

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* * *

APPENDIX - A

MEASUREMENTS OF GROWTH PARAMETERS

Samples for the batch and continuous runs were analyzed for the following:

(i) Turbidity: Turbidity was measured at 420 mμ on "Spectronic 20". Turbidity changes during growth were expressed simply as changes in optical density. At higher value of optical density (0.4 to 2.0) turbidity is not a linear function of dry weight or cell number. The sample, therefore, should be suitably diluted before measurement, in order to fall within the range of 0.0 to 0.4 optical density. In actual experiments 1:10 dilution of each sample was used for turbidity determination. With this dilution, the turbidity of all the samples fell within the range of 0.0 to 0.4 optical density unit [15].

(ii) Dry Weight: For dry weight measurement, 80 ml. of culture was taken. It was centrifuged in a high speed centrifuge at 5000 R.P.M. for 10 minutes. The supernatant was decanted off. Part of the supernatant was preserved in the refrigerator for analysis of glucose concentration. The bacterial mass was resuspended in distilled water and centrifuged again. The supernatant was decanted off. The cells were thus washed thrice with distilled water. Finally, the cell suspension was transferred to small tared weighing bottles and dried in an oven overnight at 85°C. It was then accurately weighed on an analytical balance and the dry weight of the cells in the sample calculated [15].

(iii) Viable Count: This gives the number of viable cells in the culture. Cotton plugged test tubes containing 9.0 ml. of saline solution (0.85 per cent, w/v) were sterilized in an autoclave. One ml. of the culture was then diluted serially in the saline to give 1:10, 1:100, 1:1000 etc., dilutions, respectively of the culture. Cotton plugged test tubes containing 15 to 20 ml. of sterile melted nutrient agar were kept ready at 40-45°C. One ml. of a suitable dilution of bacterial suspension was then transferred to a sterile petri dish, and the melted agar from one test tube was poured onto it. The bacterial suspension was mixed well with the melted nutrient agar by giving the petri dish a horizontal circular motion. It was allowed to solidify and placed in an inverted position in the incubator at 35°C for about 36 hours. All dilutions were plated in triplicate. The colonies were counted and the average number of cells in the original suspension was estimated. For accuracy in counting, it is desirable that the incubated agar plate should contain between 30 to 300 colonies [15].

(iv) Total Count: The method gives the total number of cells, alive and dead, in the culture. A counting chamber of the hemocytometer type was employed. The chamber consisted of a ruled slide and a cover slip constructed in a manner such that a definite known volume was delimited by the cover slip, slide and ruled lines.

The bacterial suspension should contain about 10^7 - 10^8 cells per ml. for accurate counting. This cell concentration results in 3-12 cell counts per square of the counting chamber. Ordinary hemocytometer, used for blood cell counts, was used in the experiments. The Petroff-Hauser or Helber bacterial counting chamber is

recommended since it gives superior results to those obtained with the ordinary hemocytometer [15].

(v) Glucose Estimation: For estimation of glucose concentration, the method of Folin and Wu [16] was used.

Alkaline copper reagent and phosphomolybdic acid colour reagents were prepared. Also a one per cent stock standard glucose solution in the saturated solution of benzoic acid was prepared. From this a dilute standard glucose solution containing 0.1 gm/litre or 0.1 mgm/ml. glucose was prepared fresh each time the glucose concentration was to be estimated.

Procedure

2.0 ml. of suitably diluted cell-free sample was taken in a Folin-Wu sugar tube. To this was added 2.0 ml. of alkaline copper reagent. It was mixed by lateral shaking and placed in a boiling water bath for six minutes. It was removed without shaking and cooled in a large beaker of cold water for 2-3 minutes. To this was added 2.0 ml. of phosphomolybdic acid colour reagent, was allowed to stand for few minutes until the cuprous oxide had completely dissolved and then diluted to the 25 ml. mark with distilled water. It was mixed well by repeated inversions and allowed to stand for 10-15 minutes. A portion of the coloured solution was transferred to the spectrophotometer tube and read in the "Spectronic - 20" at 500 mμ within the next 15 minutes, against a blank tube set at zero.

The blank was obtained by running a parallel determination as described above with 2.0 ml. of distilled water in place of the culture sample. The spectronic -20 was adjusted

to its zero reading against this solution. A similar determination was carried out with 2.0 ml. of standard glucose solution. The concentration of glucose in the cell free sample is then given by the following:

$$\text{Concentration of unknown} = \text{Reading of unknown} \times \frac{\text{Concentration of Standard}}{\text{Reading of Standard}}$$

* * *

APPENDIX - B

THEORETICAL CALCULATIONS BASED ON THE MONOD MODEL

According to Monod's model the equations representing batch growth are

$$\frac{dC_x}{dt} = \mu C \qquad (B.1)$$

$$\frac{dC_s}{dt} = -\frac{1}{Y}\mu C \qquad (B.2)$$

where $\mu = \mu_m \frac{C_s}{K_s + C_s}$ (B.3)

$$Y = -\frac{dC}{dC_s} = \frac{C_{si} - C}{C_{si} - C_s} \qquad (B.4)$$

and $K_s = C_s$ when $\mu = \frac{\mu_m}{2}$ (B.5)

The equations representing continuous growth are

$$\frac{dC}{dt} = \mu C - D C \qquad (B.6)$$

$$\frac{dC_s}{dt} = -\frac{1}{Y}\mu C + D(C_{si} - C_s) \qquad (B.7)$$

(i) From batch data for C_{si} = 2 gms./litre:

$$\mu_m = 0.76\ hr^{-1} \quad ; \quad Y = \frac{792}{2000-14} = 0.399$$

It is difficult to calculate K_s from batch data as it is very small and the value of μ at small values of C_s is difficult to estimate experimentally. K_s is, therefore, estimated from the continuous culture data

At steady state in the continuous culture:

$$\mu = D = \mu_m \frac{C_s}{K_s + C_s} \qquad (B.8)$$

$$Y = \frac{(C_.)_{ss}}{C_{si} - (C_s)_{ss}} \qquad (B.9)$$

$$K_s = (C_s)_{ss} \frac{\mu_m - D}{D} \qquad (B.10)$$

Now from continuous culture data for C_{si} = 2.0 gms./litre and D = 0.36 hr^{-1}, we have

$(C_.)_{ss}$ = 687 mgms/litre ; $(C_s)_{ss}$ = 24 mgms/litre

therefore, $Y = \frac{687}{2000-24} = 0.348$

$$K_s = 24.0 \times \frac{0.76 - 0.36}{0.36} = 25.7 \text{ mgms/litre}$$

and at D = 0.66 hr^{-1}

$(C_.)_{ss}$ = 655 mgms/litre ; $(C_s)_{ss}$ = 29.2 mgms/litre

therefore, $Y = \frac{655}{2000 - 29.2} = 0.332$

$$K_s = 29.2 \frac{0.76 - 0.66}{0.66} = 4.43 \text{ mgms/litre}$$

The average value of K_s from the above results is

$$(K_s)_{av.} = \frac{25.7 + 4.43}{2} = 15.06 \text{ mgms/litre}$$

Using the above values of μ_m, Y and K_s, the theoretical values of $(C_.)_{ss}$ and $(C_s)_{ss}$ are calculated from Monod's model

μ_m = 0.76 hr^{-1} ; K_s = 15.06 mgms/litre ; Y = 0.399

then at D = 0.36 hr^{-1}, we have

from (B.10), $(C_s)_{ss} = 15.06 \frac{0.36}{0.76 - 0.36} = 13.55$ mgms/litre

from (B.9) $(C_x)_{ss} = 0.399\ (2000-13.55) = 793$ mgms/litre

Similarly, at $D = 0.66\ hr^{-1}$, we have

$$(C_s)_{ss} = 15.06\ \frac{0.66}{0.76 - 0.66} = 99.5 \text{ mgms/litre}$$

and $(C_x)_{ss} = 0.399\ (2000-99.5) = 758$ mgms/litre.

These values have been tabulated in Table I.

(ii) From batch data for $C_{si} = 10$ gms/litre

$$\mu_m = 0.58\ hr^{-1} \quad ; \quad Y = 0.321$$

The value of K_s calculated in part (i) will be used here.

$$K_s = 15.06 \text{ mgms/litre}$$

Then, at $D = 0.54\ hr^{-1}$, we have

$$(C_s)_{ss} = 15.06\ \frac{0.54}{0.58 - 0.54} = 203 \text{ mgms/litre}$$

and $(C_x)_{ss} = 0.321\ (10{,}000 - 203) = 3150$ mgms/litre

These values have been tabulated in Table I.

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CHE-1970-M-SAN-BAT